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## ETIOLOGY OF OROYA FEVER.

### XIII. CHEMOTHERAPY IN EXPERIMENTAL *BARTONELLA BACILLIFORMIS* INFECTION.

By HIDEYO NOGUCHI, M.D.

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PLATES 15 AND 16.

(Received for publication, January 13, 1928.)

In the course of our studies on *Bartonella bacilliformis* infection in monkeys, we submitted several *Macacus rhesus*, in which experimental verrucous lesions had been induced by means of cultures, to treatment with chemicals which had proved therapeutically useful in spirochetal and leishmania infections. Salvarsan had already been recommended by Arce<sup>1</sup> in the treatment of malignant verruga in man. In our experiments salvarsan, neosalvarsan, bismuth lactate, esters of chaulmoogra oil, sodium gynocardate, neutroflavine, and urotropin were tested. It was not deemed suitable to test tartar emetic, since it is a drug of slow therapeutic action, and the experimental verrucous lesions in the monkey lead in any case to spontaneous retrogression.

#### *Chemical Action in Vitro.*

Although it was not expected that a relationship would be shown to exist between the action of the chemical when tested on *Bartonella in vitro* and the verrucous lesions *in vivo*, it was considered of interest to determine the direct effects of the chemicals on the bacilli. The substances to be tested were added directly to the culture media, and the cultures were incubated at 25°C. for a period of 13 days. The results are shown in Table I. Neutroflavine inhibited growth in 1:10,000,000 dilution. Formalin was almost as effective, and neosalvarsan, novasurol, and mercuric chloride were effective up to

<sup>1</sup> Arce, J., *An. Facultad Med. Lima*, 1918, i, No. 3, 21-53, 130-161; No. 4, 24-52.

TABLE I.  
*Growth-Inhibiting Properties in Vitro.*

	Final concentration of substance in culture medium					
	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Bismuth albuminate.....	— (Turbid)	— (Turbid)	++++	++++	++++	++++
Tartar emetic.....	— (Turbid)	— (Clear)	—	+	++++	++++
Neosalvarsan.....	— (Clear, deep brown)	— (Brown)	— (Yellowish)	—	++	++++
Tryparsamide.....	± (Clear)	++++	++++	++++	++++	++++
Mercuric chloride.....	(Turbid)	— (Clear)	—	—	++++	++++
Novasurol.....	— (Clear)	—	—	—	++++	++++
Mercurochrome.....	— (Deep red, clear)	— (Deep red)	— (Eosin red)	++++ (Lt. eosin)	++++ (Tr. pink)	++++
Neutroflavine.....	(Turbid, deep gold)	(Turbid, gold)	— (Greenish yellow, clear)	— (Greenish)	— (Lt. green)	— (Tr. green)
Optochin.....	(Turbid, white)	— (Opalescent)	— (Clear)	+++	+++	+++
Sodium taurocholate.....	(Turbid, brownish)	?	+	+	+++	+++
Phenol.....	— (Opalescent)	?	+++	+++	+++	+++
Formalin.....	— (Clear)	—	—	—	±	+++
Lugol's solution.....	— (Clear)	++++	++++	++++	++++	++++

— = complete inhibition of growth.  
++++ = no inhibition of growth.

1:100,000. Mercurochrome and tartar emetic required at least 1:10,000 concentration to prevent growth.

The first experiments were made on monkeys in which the cherry-red verrucous lesions on the abdominal skin and eyebrows had reached maximal size and had persisted in this state for several days. Blood cultures taken shortly before or at the time of first injection of the chemical into the circulation proved subsequently to be negative, and bits of excised nodules taken at the same time<sup>2</sup> showed few bacilli or even none at all by culture or in section. These last findings could not be known at the time of treatment, since the bacilli require 10 to 14 days to become evident in culture.

However, distinction between the ordinary or spontaneous regression of the nodules and the regression taking place after the use of chemicals, is entirely possible. The mature nodules undergo spontaneous regression slowly,<sup>3</sup> while in the animals given chemicals there occurred rapid loss of cherry-red color, usually in 24 to 48 hours after the first injection, followed by a still more rapid reduction in size most pronounced in the nodules located in the abdominal skin. At the expiration of 5 to 6 days small pale fibrous areas alone remained to indicate the site of the nodules, and in 10 to 14 days all vestiges had disappeared, the lesions of the eyebrows persisting somewhat longer than those of the abdomen. The protocols of these experiments follow.

### *Protocols.*

*Macacus rhesus* 1-T, injected intravenously on Oct. 14, 1926, with 0.5 cc. of a mixture of

4 cc. defibrinated blood (culture + + +) from *M. rhesus* 54 (P. 5 strain<sup>4</sup>),

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown on leptospira medium for 72 hours,

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown for 6 days on blood agar slants.

In addition, 4 intradermal injections of the mixture were made on the left abdominal wall and 2 on the left eyebrow. Also nodular tissue freshly excised from *M. rhesus* 54 was applied to scarified areas on the right abdominal wall and right eyebrow.

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<sup>2</sup> Ether anesthesia was used in all the operations.

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1927, xlv, 455.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.



Experimental nodules appeared on the left eyebrow in 14 days. Within a month large nodules were present on the abdomen at the sites of intradermal inoculation, and the scarified areas showed the characteristic miliary lesions. On Nov. 23 (40 days after inoculation) the hemoglobin was 35 per cent (Sahli), and the red cells 2,502,000, and there had been no fever. 0.05 gm. of salvarsan was given intravenously. Within the following week the nodules grew small and became paler. On Dec. 3 a second injection of 0.05 cc. salvarsan was administered. Within the week the nodules had become very small and pale, the erythrocytes rose to 5,600,000 and the hemoglobin to 80 per cent. When the animal was sacrificed on Dec. 13, *Bartonella bacilliformis* could not be detected in the nodular tissue either microscopically or by culture, and blood, lymph nodes, and spleen also failed to yield cultures. Sections of the nodules showed fibrous tissue.

*Macacus rhesus* 2-T. Inoculated at the same time as No. 1-T, and in the same manner, except that the eyebrows were spared. On Oct. 19 the culture titer of the blood was 1:100,000. On Nov. 15, or 32 days after the inoculation, the animal showed large mature subcutaneous nodules on the left abdominal wall and numerous red miliary lesions on the scarified areas on the right side (Fig. 1). Blood cultures made on this day later proved to be negative. A mixture of 1 cc. of 1 per cent bismuth lactate, 1 cc. of 1 per cent neuroflavine, and 1 cc. of 1 per cent urotropin was injected intravenously. The temperature rose to 105.2°F. on the following day (Nov. 16), but the animal appeared well. On Nov. 23, a second injection of the same mixture was given. The nodules soon became bluish, smaller in size, and continued to diminish in volume after the second dose of the drugs (Fig. 2). On Dec. 3 a third injection was given. During the following 9 days the nodules became very small and pale (Fig. 3). Nodules and spleen removed on Dec. 16 did not yield *Bartonella bacilliformis* in culture.

*Macacus rhesus* 3-T, inoculated in same manner as *Macacus* 2-T. By Nov. 24 the abdomen showed large, mature subcutaneous nodules (Fig. 4), and large bluish red nodules on each leg where the injections had been made into the saphenous veins. A general miliary eruption was also present on the abdomen (Fig. 5). The hemoglobin was 45 per cent (Sahli), and the red blood cells 3,960,000. No fever. A suspension of one of the nodules yielded cultures of *Bartonella bacilliformis* in a dilution of 1:1,000, but the blood proved negative, although the titer had been 1:100,000 on Oct. 19 and Nov. 3. At this time (i.e., on Nov. 24, 41 days after inoculation), 1 cc. of moogrol<sup>5</sup> was given intravenously. Within 48 hours the nodules had become bluish in color and somewhat smaller. Fig. 6 shows the appearance of the nodules 9 days after the first treatment. On Dec. 3 another injection of 1 cc. of moogrol was given. The temperature rose on the 2 days following to 104.4-104.6°F. By Dec. 13 the nodules had contracted considerably (Fig. 7). The spleen was negative for culture on Dec. 16, and all lesions had disappeared by Dec. 30. The blood culture was negative a week later.

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<sup>5</sup> Burroughs Wellcome and Company's preparation of the esters of chaulmoogra oil.

*Macacus rhesus* 4-T was inoculated intravenously on Nov. 24, 1926, with 1 cc. of a mixture of cultures of *Bartonella bacilliformis*, besides which a suspension of an abdominal nodule of *M. rhesus* 3-T was given intravenously and introduced intradermally into both eyebrows and the abdominal skin. On Dec. 21 the blood cultures were positive for *Bartonella bacilliformis* in a 1:100,000 dilution. The nodules were fully developed by Dec. 28, 34 days after inoculation (Figs. 8 and 11), when the first intravenous injection of bismuth lactate, proflavine, and urotropin was given (a mixture of 1 cc. of a 1 per cent solution of each). Blood cultures were negative at this time. The temperature rose to 105°F. on the day following the treatment, and the nodules had already shrunk and become paler. A double dose was given on Dec. 30, and again on Jan. 3, 1927. No febrile reactions. Blood culture was negative on Jan. 5, 1927. The hemoglobin was 78 per cent (Sahli), and the erythrocytes 4,400,000. Cultures made from nodules and lymph nodes on Jan. 10, 1927, were negative. The lesions regressed rapidly (Figs. 9 and 12), and only fibrous traces remained on Jan. 28, 1927 (Figs. 10 and 13).

*Macacus cynomolgus* 5-T, inoculated intradermally with a suspension of nodular tissue from *M. rhesus* 1-S<sup>6</sup> into the right eyebrow and the abdominal skin on Dec. 7, 1926. This animal remained afebrile but developed large cherry-red nodules on eyebrows and abdomen by Dec. 27. The blood culture titer was 1:10 on Jan. 6, 1927. On Jan. 8 and 14, 1927, or 32 and 38 days after inoculation, an intravenous injection was made of 1 cc. of 5 per cent bismuth lactate, 1 cc. of 5 per cent urotropin, and 1 cc. of 1 per cent neutroflavine. Figs. 14 and 15 show the appearance of the lesions on Jan. 7. The nodules began to shrink in the following week and within 2 weeks had become small and fibrous. The appearance on Feb. 8 is shown in Figs. 16 and 17.

The next step was to test the action of the chemicals on the appearance and development of the nodules in instances in which the chemicals were administered *before* the lesions reached full development, *i.e.*, 2 to 3 weeks after the inoculation of the infective material into the skin, while the lesions were growing in size daily. Under these circumstances the drugs failed to influence the progress of the lesions, which in one instance attained unusually large proportions (Figs. 19, 20). The usual cherry-red color developed without hindrance, and the scarified areas became covered with the characteristic deep red miliary nodules. As in untreated animals, the skin of the lower half of the abdomen became edematous.

<sup>6</sup> Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 821.

*Protocols.*

*Macacus rhesus* 6-T, inoculated intravenously (2 cc.) and intradermally on Dec. 28, 1926, with a mixture of cultures of *Bartonella bacilliformis* and a suspension of nodular tissue of *M. rhesus* 4-T. The nodules were well advanced on Jan. 24, 1927, 27 days after the inoculation, when intravenous injection of a mixture of bismuth, urotropin, and proflavine (1 cc. of 1 per cent proflavine, 1 cc. of 5 per cent urotropin, and 1 cc. of 5 per cent bismuth lactate) was begun. Blood taken just before the treatment yielded cultures in a dilution of 1:10. No change was observed in the nodules after the first injection. The second injection, given on Jan. 26, 1927, was followed by slight diminution in the size of the nodules on the eyebrows while the abdominal lesions continued to enlarge.

*Macacus rhesus* 7-T, inoculated by scarification and intradermal injection on Mar. 8, 1927, with a suspension of the nodule from *M. rhesus* 3-A, which had been infected by means of the L<sub>8</sub> strain of *Bartonella bacilliformis*.<sup>7</sup> Small nodules were present 20 days after inoculation (Fig. 18), when the animal was given the first intravenous injection of 0.1 gm. of neosalvarsan. The injection was repeated 2 days later. The nodules continued to grow gradually and within 2 weeks they attained unusually large dimensions (Figs. 19 and 20). Certainly no inhibitory action was apparent.

*Macacus rhesus* 8-T. This animal was inoculated in the same manner and on the same date as the foregoing monkey. Nodules had appeared by Mar. 28 (Fig. 21), when an intravenous injection of 2 cc. of 3 per cent sodium gynocardate and an intramuscular injection of 0.15 cc. of chaulmestrol<sup>8</sup> were given. This was followed 2 days later by an injection of 3 cc. of the sodium gynocardate and an injection of 0.2 cc. of chaulmestrol. No inhibitory effect upon the development of the cutaneous lesions was apparent and the large nodules are shown in Figs. 22 and 23.

## SUMMARY.

The therapeutic effect of several antiparasitic chemicals on experimental verruga peruana is described. The drugs were administered by intravenous injection according as the nodules (1) were already developed to an approximate maximum, or (2) were still in the active period of growth.

The effect of the drugs was different under the two circumstances of

<sup>7</sup> Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 219.

<sup>8</sup> The name given by the Winthrop Chemical Company to their preparation of the esters of chaulmoogra oil, of which the Company kindly furnished a sample.

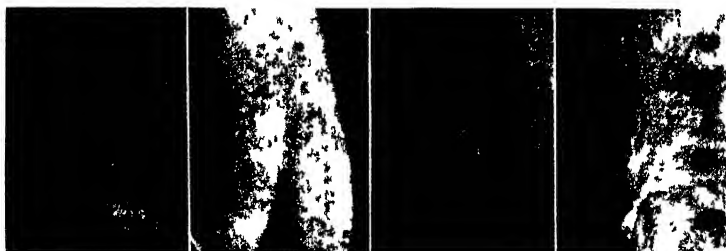
their administration. When they were given after the maturity of the nodules they hastened the regressive process, but when given during active growth of the lesions no action whatever was detected.

*Bartonella bacilliformis* in culture is acted upon injuriously by a number of the chemicals employed in the therapeutic tests, the most active being formalin and neutroflavine.

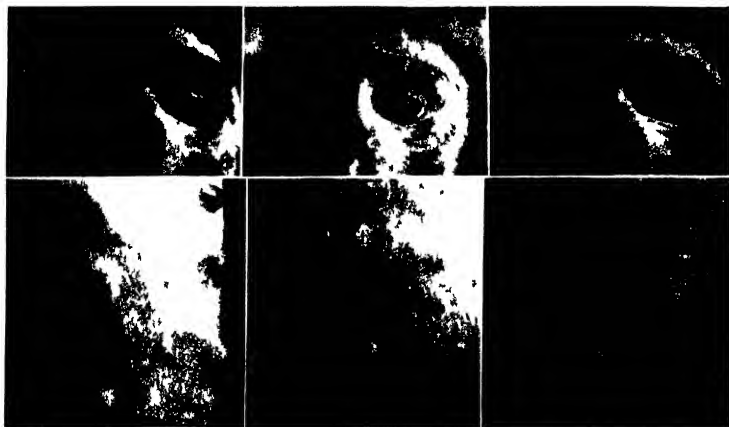


*M. rhizus* 2 I

Before treatment (32 days after inoculation)      17 days after treatment      25 days after treatment  
(One nodule removed for study 32 days after inoculation)

*M. rhizus* 3 I

Before treatment      Nodular and milium eruptions 41 days after inoculation      9 days after treatment      19 days after treatment  
(One nodule removed for study 41 days after inoculation)

*M. rhizus* 4 I

Before treatment      13 days after treatment      38 days after treatment  
Nodules on eyebrows and abdomen 34 days after inoculation      (One nodule removed for study 34 days after inoculation)



*M. cynomolgus* 5-T.

Before treatment (32 days after in-  
oculation).

31 days after treatment was begun.

*M. rhesus* 7-T.

Before treatment (20  
days after inoculation).

15 days after treatment was begun.

*M. rhesus* 8-T.

Before treatment (20 days  
after inoculation).

8 days after first treat-  
ment.

15 days after treatment  
was begun.





## PHLEBOTOMUS AND OROYA FEVER AND VERRUGA PERUANA.

By HIDEYO NOGUCHI, RAYMOND C. SHANNON, EVELYN B. TILDEN, AND JOSEPH R. TYLER.

*(From The Rockefeller Institute for Medical Research and the Rockefeller Foundation.)*

HISTORIANS of the early seventeenth century, writing of the conquest of the Incas, refer to the Peruvian Indians as suffering from numerous warts ("verrugas"), varying in size from small red prominences to masses as large as eggs, and covering the face and limbs. Many of Pizarro's soldiers developed the warty condition and died of the fever sometimes attending it. In their ignorance they attributed death not to the peculiar disease but to fish or water supposed to have been poisoned. In earlier times the disease seems to have occurred in regions now in Ecuador, but at present it is confined to the provinces of Lima, Ancachs and Libertad in Peru, lying in south latitude 9° to 13°.

The distribution of the disease is curious. The districts at sea-level and 25 to 35 miles inland are free from it, but as the mountainous parts are approached the disease makes its appearance, and foci or endemic centers are encountered at altitudes of 9,000 feet and over. In certain narrow clefts, called "quebradas," the disease has prevailed in a severe form from early periods, and strangely enough a given village may be severely ridden and a neighboring one a few miles distant may escape entirely.

In 1870, during the construction of the trans-Andean railway, an acute, febrile and fatal disease carried off many thousand laborers in the region between Lima and Oroya. One feature of this destructive malady was an anemia so profound as to have blanched the color of the natives or, in local language, to have changed "blacks" into "whites." Another curious circumstance noted at this time was that the workmen escaped the disease so long as they avoided certain localities at night. A single night passed in these danger zones might

be followed by fever and death. Finally, the laborers were removed from them before sunset, and after this was done the disease abated.

This severe disease of the Andes was called Oroya fever, and while it might run its fatal course without other symptoms than fever and anemia, yet at times it was attended by the warty skin affection previously mentioned; or, when recovery occurred, the "verrugas" might appear. Conversely, sometimes the warty disease arose attended only with mild fever and anemia. Because of their frequent association in the same individual and their common geographical distribution, the two conditions, Oroya fever and verruga, came to be regarded in the popular mind as one disease appearing in a malignant and in a benign form.

The identity of the two forms was, however, a controverted point, and to settle it a medical student, David Carrion, in 1885, inoculated himself on both arms with tissue juice taken from "verrugas." He developed Oroya fever and died. Since this self-sacrificing experiment the malady has often been called Carrion's disease. In 1905 a Peruvian physician, Barton, discovered in the red blood corpuscles of Oroya fever patients certain rod-shaped bodies resembling bacilli, and later similar rods were detected in small numbers in the blood of verruga patients. These bodies were named *Bartonella bacilliformis* by the Commission of the Harvard School of Tropical Medicine in 1913 and regarded by them not as bacterial but as protozoal organisms. Many attempts were made to develop the rods in artificial cultures but without success. Such bacteria as were cultivated proved to be either secondarily invading organisms (paratyphoid bacilli) or extraneous contaminants. That the verrucous disease can be transmitted by inoculation from man to monkey has been known since 1909, but the Harvard Commission was unable to detect any of the rod-shaped organisms either in the human warts or in those induced in animals.

In 1925 Dr. T. S. Battistini, a Peruvian fellow of the Rockefeller Foundation, brought to one of us (Noguchi) a specimen of blood taken from a case of Oroya fever. The red corpuscles of the blood contained the rods, and cultivation experiments were undertaken which resulted successfully (Noguchi). The microorganisms obtained in the cultures reproduced on inoculation into monkeys and apes experimental diseases agreeing with both Oroya fever and verruga. Subsequently

the microorganism was isolated from the blood of two other cases of Oroya fever and seven of verruga peruana, and from the skin nodule in a case of verruga (Noguchi). The bacterial incitant of the disease was now isolated, and the important fact determined that, as grown outside the body, it was capable of infecting monkeys, in which could be induced the two characteristic manifestations of Carrion's disease.

The one essential point which remained to be established in order to account for the origin of the disease was the mode of infection. Indications pointing to direct transfer from person to person were wanting, while evidence implicating insects in the transmission existed. The strict limitation of the endemic zone of the disease and the nocturnal dangers pointed to an insect source of inoculation. This aspect of the subject had been investigated in 1913 with remarkable energy and penetration by Charles H. T. Townsend, an American entomologist. He gave consideration to all kinds of blood-sucking insects to be found in the verruga zone, and, after excluding one and another variety of insects, finally, in a brilliant manner, concentrated attention on certain blood-sucking gnats of the class *Phlebotomus*. By excluding all insects whose range extends outside the verruga zone, he reduced the possible carriers to buffalognats, horseflies and phlebotomi, and by excluding the insects which bite by day as well as by night, he reduced the possible verruga vectors to phlebotomi alone. So certain did he feel of his discovery that he called the gnat "*Phlebotomus verrucarum*." Townsend went one step further in attempting experimental induction of verruga in dogs and man by means of phlebotomi. It is doubtful whether he actually succeeded in this undertaking.

With the artificial cultivation of the rods, the mode of transmission was opened to rigid experimentation. One of us (Noguchi) discovered that *Bartonella bacilliformis* can be taken up by the wood tick from the blood of an infected monkey and be transferred to a healthy monkey through bites. Probably this is a purely mechanical process without significance so far as the natural insect vector of Carrion's disease is concerned. The next step was actually to test insects from the verruga zone in Peru. The International Health Division of the Rockefeller Foundation was invited by the Department of Health of Peru to send an entomologist to Peru, and one of us (Shannon) spent five months there studying insect life in the verruga zone and collecting

and sending to the Rockefeller Institute insects for purposes of inoculation, according to a plan arranged by Dr. Noguchi before he sailed for Africa to study yellow fever. The phlebotomi collected and identified consisted of *Phlebotomus verrucarum* Townsend and two new species named respectively *P. noguchii* and *P. peruensis*, which are readily differentiated from each other and from *P. verrucarum* by the sex characters of the males. Only the females are blood-suckers, however, and those of *verrucarum* and *noguchii* are indistinguishable, hence some doubt must remain whether the females of both species carry *Bartonella bacilliformis*. We are confident that the females of *P. noguchii* are carriers, and we think that *P. peruensis* is probably not a carrier.

Other blood-sucking insects which were tested by inoculation are ticks (Argasidae), mites (Trombidium), midges, sheep lice, bird lice, bedbugs, mosquitoes (*Anopheles pseudopunctipennis* and *Culex fatigans*), buffalognats (*Simuliidae*), flies (*Stomoxys calcitrans*), "sheep ticks" (*Melophagus ovinus*), Streblidae of blood-sucking bats, and fleas of guinea-pig and dog. None of these was found to harbor *Bartonella bacilliformis*.

The plan followed was to inject saline suspensions of the crushed insects into the skin of *rhesus* monkeys. No verrucous nodules developed. At regular intervals thereafter cultures were made from the blood of the inoculated monkeys with a view to determining whether the *Bartonella* had entered the blood and multiplied in it. Four different lots of phlebotomi, as tested in this way, were proved to carry *Bartonella bacilliformis*. In the first instance the culture was obtained with blood withdrawn from the inoculated monkey on the 19th day, in the second on the 20th day, in the third on the 10th day, and in the fourth on the 42nd day. The inoculation of monkeys with the cultures thus obtained produced experimental verrucous nodules, with recovery of the *Bartonella* from the blood and the nodular tissue. The chain of evidence uniting *Phlebotomus* with Oroya fever and verruga peruana may be said to have been completed by these tests.

## STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

### XI. AN INQUIRY INTO THE MODE OF ACTION OF ANTIBACTERIOPHAGE SERUM.

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That sera of animals immunized by parenteral introduction of lytic filtrates have the property of neutralizing bacteriophage has been recognized since the work of Bordet and Ciuca in 1921 (1), but this demonstration of a new antigen was at the time of purely academic interest. In 1925, however, Sonnenschein (2), and shortly afterward Katsu (3), stated that human blood possessed antibacteriophagic properties in relation to certain infectious diseases, and they suggested that the reaction might be of diagnostic value. This possibility has made the serological study of bacteriophage of considerable practical importance.

#### *Methods.*

*Purification of Phages.*—It is a common observation that when a lytic filtrate is first obtained it ordinarily contains a number of different principles, or bacteriophages. The first procedure in the purification of this mixture is to carry lysis in series through a number of passages on the bacterial strain, thus eliminating by dilution any phages that act only on heterologous bacteria.

It is probable that even the filtrate thus obtained still consists of several different components which act on the same bacterial species, and which can be further separated by the plating method. For each phage forms a characteristic plaque, as has been demonstrated by Bail (4) and by Gratia (5). Plaques of the same sort should be picked for several passages, in this manner securing a pure phage.

Pure phages obtained in this manner were used as antigens. Immunization was conducted by intravenous and subcutaneous injections, and in both cases neutralizing sera were obtained. The sera of mice, on the other hand, that were fed phage daily for 6 weeks, were not capable of neutralizing the phage ingested.

*Technique for Demonstrating Neutralization.*—Bordet and Ciuca (1) demonstrated neutralization by adding a mixture of a specific serum and the appropriate phage to a culture of susceptible bacteria in broth, the absence of visible lysis serving as a criterion of neutralization. They also demonstrated (6) in a control experiment, that normal serum did not prevent phage action. D'Herelle and Eliava (7), using this technique, found that on prolonged incubation lysis took place, and so concluded that the serum merely inhibited the lysis, but that it did not inactivate the phage. Wagemans (8), however, found that if the time of contact between phage and serum was sufficiently long, neutralization was complete and the phage could not be regenerated. A technique commonly employed (9) has been to mix phage and serum for a varying length of time and then to streak this mixture on a plate seeded with susceptible bacteria, neutralization

TABLE I.  
*The Neutralization of Coliphage by Immune Serum.*

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C.	Normal serum P.C. coliphage	<i>B. coli</i> “ “	10 <sup>-9</sup>	+++	++	100±	—
			10 <sup>-4</sup>	—	—	—	—

+++ indicates complete lysis.

++, +, indicate lesser degrees of lysis.

Numbers indicate the number of plaques.

± following a number indicates that the number of plaques was estimated.

— indicates absence of lysis.

These signs are used in all of the following protocols in which lysis on agar slants is recorded.

being demonstrable by the absence of plaque formation. Sonnenschein (10) did this in a quantitative manner, mixing diluted phage with undiluted serum on the one hand, and undiluted phage with diluted serum on the other, running drops of the mixtures down the previously inoculated surface of an agar plate and observing lysis after incubation.

In our search for a satisfactory technique the findings of Wagemans (8) were first repeated and confirmed. It was found, however, that the time required for complete neutralization depends on the potency of the serum, a serum of high potency causing complete neutralization in a comparatively short time. Inhibition of lysis in broth proved unsatisfactory as a method, since a partial neutralization can delay or prevent visible lysis, although increase of the phage may proceed at a fairly rapid rate. For detecting partial neutralization it has been found

TABLE II.  
The Specificity of Antiphage Sera.

Phage	Antigen of serum	Bacterium	Dilution of phage				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C.	{ Normal P.C. phage Laudman phage P. I. D. " B.H. " }	<i>B. coli</i>	$10^{-8}$	+++	++	+	14
			$10^{-6}$	—	—	—	—
			$10^{-8}$	+++	++	+	11
			$10^{-10}$	+++	++	+	5
			$10^{-10}$	+++	++	+	8
	{ Normal P.C. phage Laudman phage P.I.D. " B.H. " }	<i>B. dysenteriz</i> Shiga	$10^{-8}$	+++	+++	+	15
			$10^{-6}$	—	—	—	—
			$10^{-8}$	+++	+++	+	20
			$10^{-8}$	+++	+++	+	25
			$10^{-8}$	+++	++	+	6
Laudman	{ Normal P.C. phage Laudman phage P.I.D. " B.H. " }	<i>B. coli</i>	$10^{-10}$	+++	++	+	3
			$10^{-8}$	+++	++	+	4
			$10^{-8}$	—	—	—	—
			$10^{-10}$	+++	++	+	—
			$10^{-8}$	+++	++	+	3
	{ Normal P.C. phage Laudman phage P.I.D. " B.H. " }	<i>B. dysenteriz</i> Shiga	$10^{-8}$	+++	+++	++	16
			$10^{-10}$	+++	+++	++	7
			$10^{-8}$	—	—	—	—
			$10^{-10}$	+++	+++	++	8
			$10^{-8}$	+++	+++	++	—
P.I.D.	{ Normal P.C. phage Laudman phage P.I.D. " B.H. " }	M.T. <sup>2</sup>	$10^{-8}$	+++	++	+	12
			$10^{-8}$	+++	++	+	—
			$10^{-8}$	+++	++	+	7
			$10^{-8}$	—	—	—	—
			$10^{-7}$	+++	++	+	10
B.H.	{ Normal P.C. phage Laudman phage P.I.D. " B.H. " }		$10^{-8}$	+++	++	100±	1
			$10^{-8}$	+++	++	100±	—
			$10^{-8}$	+++	++	100±	—
			$10^{-8}$	+++	++	100±	3
			$10^{-8}$	—	—	—	—



convenient to drop a mixture of phage and antiserum on an evenly inoculated agar surface, and to compare the resulting lysis with that caused by a mixture of phage and normal serum. Absence of lysis under these conditions, however, does not necessarily indicate complete inactivation of the phage.

As the reaction of neutralization may at times continue for several days, it was found best to limit the time of contact of phage and serum to a fixed period and then to determine quantitatively, by the titration method of Appelmans (11), the amount of phage remaining active. In addition to this, serum was mixed with serial dilutions of the phage, and after the given period of contact a drop of each

TABLE III.  
*The Specificity of Neutralization of Adapted Phages.*

Phage	Antigen of serum	Susceptible bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. phage adapted to Shiga bacillus	{ Normal P.C. phage Laudman phage }	<i>B. coli</i>	10 <sup>-8</sup>	+++	++	+	2
			10 <sup>-9</sup>	—	—	—	—
			10 <sup>-8</sup>	+++	++	+	11
	{ Normal P.C. phage Laudman phage }	<i>B. dysenteriae</i> Shiga	10 <sup>-8</sup>	+++	++	100±	2
			10 <sup>-9</sup>	—	—	—	—
			10 <sup>-8</sup>	+++	++	100±	1
Laudman phage adapted to <i>B. coli</i>	{ Normal P.C. phage Laudman phage }	<i>B. coli</i>	10 <sup>-10</sup>	+++	+++	150±	5
			10 <sup>-9</sup>	+++	+++	150±	5
			10 <sup>-8</sup>	—	—	—	—
	{ Normal P.C. phage Laudman phage }	<i>B. dysenteriae</i> Shiga	10 <sup>-10</sup>	+++	+++	+	14
			10 <sup>-10</sup>	+++	+++	+	20
			10 <sup>-4</sup>	—	—	—	—

of these mixtures was run down the surface of agar slants previously inoculated with the susceptible bacterium. The results with immune serum were then compared with those with normal serum. A typical protocol is given in Table I, which shows the neutralization of P.C. coliphage by its specific antiserum.

#### EXPERIMENTAL.

*Specificity of Neutralization.*—Maisin (12), in 1921, stated that antilytic sera are not specific in their action, but this finding lacks confirmation. In most of the experiments here to be reported four bacteriophages were used. They are listed below and their respective activity is indicated.

1. P.C. coliphage, acting on *B. coli* and *B. dysenteriae* Shiga.
2. Laudman Shiga phage, acting on *B. coli* and *B. dysenteriae* Shiga.
3. P.I.D. M.T.<sup>2</sup> phage, acting on M.T.<sup>2</sup> (*B. pestis caviar*).
4. B.H. staphylococcus phage, acting on *Staph. aureus* "G."

In addition to these, B.W. coliphage, acting on *B. coli*, was used in some experiments. Neutralizing sera were prepared for each of the phages, and then the ability of each antiserum to neutralize each of the phages was determined. The result of these studies is given in Table

TABLE IV.  
*Specificity of Antisera for Different Phages Acting on the Same Bacterium.*

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. coli-phage	Normal	<i>B. coli</i>	10 <sup>-9</sup>	+++	++	+	14
	P.C. phage	" "	10 <sup>-8</sup>	—	—	—	—
	Laudman phage	" "	10 <sup>-9</sup>	+++	++	+	11
	B.W. " "	" "	10 <sup>-9</sup>	+++	++	+	12
Laudman coliphage	Normal	" "	10 <sup>-10</sup>	+++	+++	150±	5
	P.C. phage	" "	10 <sup>-9</sup>	+++	+++	150±	5
	Laudman phage	" "	10 <sup>-4</sup>	—	—	—	—
	B.W. " "	" "	10 <sup>-9</sup>	+++	+++	150±	10
B.W. coli-phage	Normal	" "	10 <sup>-7</sup>	+++	++	4	—
	P.C. phage	" "	10 <sup>-7</sup>	+++	++	2	—
	Laudman phage	" "	10 <sup>-8</sup>	+++	++	3	1
	B.W. " "	" "	10 <sup>-4</sup>	1	—	—	—

II, in which it is seen that each serum exerts a specific neutralizing action on its own antigen, though it is without influence on the other phages.

The specificity of neutralization after the phage had been adapted to another bacterium was next studied. Seiffert (13) found neutralization to be specific after adaptation. Wagemans (8), Wolff and Jansen (14), and Kasarnowsky and Tiomkin-Schukoff (15), on the other hand, found that while specific neutralization usually occurred, this was not invariably the case. The results of our inquiry into this

action of the serum on the bacteria, and not to a process of neutralization of phage.

We have investigated the question thus brought up, and have used not only agglutinating sera, but also precipitating sera prepared against autolyzed broth cultures of bacteria. The results are given in Table V, which shows that no neutralizing antibodies were present in the sera.

However, when lysogenic cultures were used as antigens the antisera obtained exhibited definite antilytic properties. This experiment was carried out as follows:

Bacteria were made artificially lysogenic by continuing incubation after lysis in broth, and inoculating the overgrowth of resistants directly onto agar slants. When animals were immunized with these cultures, their serum, in addition to its agglutinating properties, became capable of neutralizing the phage carried; but here, of course, phage was also present in the antigen.

*Presence of Other Antibodies in Antiphage Sera.*—Since the filtrates used as antigens contained, in addition to the active bacteriophage, bacterial protein liberated during growth and subsequent lysis, the appearance of antibacterial antibodies was to be expected. These appeared with regularity, and antilytic sera prepared by immunization of animals with crude filtrates of lysed cultures regularly formed a precipitate when mixed with autolysates of the homologous bacterium, or with the filtrate used as antigen. Bruynoghe and Dubois (21) have found that precipitation is to some extent specific for the bacteriophage. In our hands the method has been unsatisfactory, since with the presence of degradation products of bacteria of closely related species there is enough non-specific precipitation to obscure the results.

The presence of agglutinins in antilytic sera is usual (22, 23). Marshall (24), however, prepared a serum that did not agglutinate the homologous bacterium. The results of our studies have been variable. From Table VI it will be seen that sera against the coliphage and the Shiga phage did not agglutinate the homologous bacteria, while those against M.T.<sup>2</sup> phage and staphylococcus phage were definitely agglutinating. In the case of staphylococcus agglutination occurred with normal sera also, so that the results are not so striking as the truly specific reactions obtained with M.T.<sup>2</sup>

Complement fixation was studied by Sanderson (25) and later by Flu (26). Their findings showed that with rabbit serum complement is fixed non-specifically in the presence of bacterial protein and the reaction is not related to the presence of bacteriophage; occasionally complement may be fixed even with plain broth. In view of these reports complement fixation was not tested by us.

*Independence of the Two Types of Antibodies.*—That the antibodies against the bacteria and those against the phage are distinct can be readily demonstrated by the absorption test. The antibodies against the bacteria may be absorbed, leaving unreduced the antilytic power of the serum. This fact has been observed in several laboratories (23, 27). It is also possible to remove a precipitate resulting from a mixture of a bacterial autolysate and antilytic serum without reducing the neutralization titer of the serum.

*The Reaction of Neutralization.*—In order to get some conception of the nature of the reaction when bacteriophage is neutralized by antiserum, a study of the factors entering into the reaction was made.

(a) *Rate of Neutralization.*—When phage and antiserum are placed in contact with each other, neutralization proceeds at first rather rapidly, but the rate of this process gradually decreases. Seisser (28) found that the greater part of the phage is neutralized within 24 hours, and Wagemans (8) found that complete neutralization may require as long as 4 days. Higher temperatures, according to Otto and Munter (29), increase the rate of neutralization. This was confirmed by Arnold and Weiss (30).

All of these findings have been confirmed in our laboratory.

Arnold and Weiss found that neutralization follows the law of multiple proportions and also reported the Danysz-Bordet phenomenon (30).

(b) *The Rôle of Complement.*—Osumi (31) found complement necessary for neutralization of bacteriophage by immune serum, but this finding has not been confirmed by other workers, and in our experiments complement played no part in the reaction.

(c) *Mode of Neutralization.*—Seiffert (13) reported that neutralization proceeds by a reduction in size, rather than number, of the plaques. Grigorieff (32), on the contrary, found that serum acts by inactivating individual units. With the material used in the present

work the latter has been found to be the mode of action. Reduction in size of the plaques has not been observed.

(d) *Nature of the Reacting Substances.*—That the action of the serum is directly on the bacteriophage and not on the bacteria or bacterial products has been demonstrated in the preceding pages by the specificity of their neutralization, by the failure of antibacterial sera to affect them, and by the undiminished antilytic activity of neutralizing sera after the complete removal of antibacterial antibodies.

(e) *The Influence of Adsorption.*—There is a possibility that phage may be inactivated by adsorption onto a precipitate which is formed when antiserum is combined with a filtrate containing bacterial protein. This possibility was tested by means of attempts to remove phage from a filtrate by the formation of a heterologous precipitate. Horse serum and homologous immune rabbit serum were mixed in the presence of phage and incubated overnight. The tube was then centrifuged and the clear supernatant fluid titrated. The lytic activity was found equal to that of another sample of phage similarly diluted with normal serum. Moreover, precipitation of filtrates by antibacterial sera failed to reduce the titer of phage. For these reasons it seems unlikely that adsorption can be the explanation of neutralization.

(f) *Reversibility of the Neutralization.*—The view that the neutralization of phage is analogous to the neutralization of toxin by antitoxin has been offered by certain workers who present evidence that the combination can dissociate. Otto and Munter (29) reported that dilution dissociated the combination but their method does not exclude the possibility of incomplete neutralization as the explanation. Weiss (33) has reported the complete dissociation of a neutral mixture by tryptic digestion.

We have attempted, though without success, to effect dissociation (1) by changing the reaction, and (2) by digestion with trypsin.

1. *Dissociation by Changing the Reaction.*—After determining the activity of a serum it was mixed with phage in the proportion to secure a neutral mixture (in this case 1-10). The resulting inactive fluid was diluted 1-5 in buffers of various degrees of acidity or alkalinity and these mixtures were left at room temperature overnight, when they were tested for phage. Phage alone, and serum alone similarly diluted and treated in an identical manner, were found to be unaffected. Table VII shows that dissociation was not secured by this method.

2. *Dissociation by Digestion with Trypsin.*—Trypsin solution was prepared by suspending 2 per cent of Fairchild's trypsin in borax-boric acid buffer at pH 7.4, and placing the flask in the ice box overnight. This material was then filtered through a Berkefeld V candle, and the reaction adjusted colorimetrically with

TABLE VII.

*Attempt to Dissociate a Neutral Phage-Antiphage Mixture by Changing the Reaction.*

pH of buffer	4 05	4 99	5 94	6 43	7 03	7 54	7 96	8 97	9 90
Quantity of buffer, cc	0 8	0 8	0 8	0 8	0 8	0 8	0 8	0 8	0 8
Quantity of neutral mixture, cc	0 2	0 2	0 2	0 2	0 2	0 2	0 2	0 2	0 2

Mixtures left at room temperature overnight

Titer	Neg	Neg	Neg	Neg	Neg.	Neg	Neg	Neg	Neg
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TABLE VIII.

*Effect of Trypsin on Neutral Phage-Antiphage Mixture*

Day of incubation	Immediate	1	2	4	5	6
Phage with normal serum	$10^{-8}$	$10^{-8}$	$10^{-8}$	$10^{-8}$	$10^{-7}$	$10^{-8}$
" " immune "	Neg	Neg	Neg	Neg	Neg	Neg

TABLE IX.

*Effect of Trypsin on Antiphage Serum.*

Serum	Undiluted		1-100	1 10 000	1-1,000,000
	Titer	Slant	Slant	Slant	Slant
Normal	$10^{-9}$	+++	++	150±	4
Immune before digestion	Neg	—	—	—	—
Digested 1 day	$10^{-6}$	+	25±	—	—
" 2 days	$10^{-7}$	++	100±	1	—
" 4 "	$10^{-7}$	++	150±	1	—
" 6 "	$10^{-7}$	++	100±	—	—

sterile sodium hydroxide to a pH of 7.4 One volume of this solution was added to three volumes of a neutral mixture of coliphage with the corresponding anti-serum. Phage with normal serum was similarly treated as a control, and the two tubes were placed in the incubator at 37°C The solution of trypsin was shown

to be active in a duplicate tube to which a Mette tube was added. The activity of the phage in each of the two tubes was determined at intervals (Table VIII). At the same time, immune serum was similarly digested with trypsin and its ability afterwards to neutralize phage was determined at the same intervals (Table IX).

The neutralizing power of the serum alone was markedly diminished by the trypsin, but, in spite of this, the phage was not liberated from a neutral mixture with this same serum by the action of trypsin. These experiments were repeated with other phages and antisera, but no definite evidence of dissociation was obtained.

That neutral mixtures may dissociate under certain conditions is suggested, however, by the fact that when rabbits are immunized with a neutral mixture of phage and immune rabbit serum, their serum becomes capable of neutralizing phage.

*Stability of the Bacteriophage Antigen.*—Arnold and Weiss (34), and Asheshov (35) have shown that phage inactivated by heat is still antigenic. We have confirmed this. However, boiling, or autoclaving at 120°C. for 1 hour renders the phage antigenically inert, as determined by the usual course of immunization. After inactivation of phage by formalin, by immune serum, or by adsorption onto dead bacteria, its antigenic property remains unchanged.

*The Occurrence of Neutralizing Antibodies in Disease.*—As stated previously, Sonnenschein (2) and Katsu (3) detected antilytic properties of human sera and related these findings to specific infections. The subject has been studied experimentally in this laboratory by feeding the bacteria of mouse typhoid (M.T.<sup>2</sup>) to mice and testing the serum of those animals surviving the infection for neutralizing antibodies. Three phages acting on this bacterium were available, and none of these was neutralized by the serum of the surviving mice. Moreover, the spontaneous appearance of neutralizing antibodies in experimental animals has never been observed in this laboratory.

#### DISCUSSION.

The reaction between bacteriophage and specific neutralizing anti-serum constitutes a definite serological phenomenon, different from and independent of any of the known reactions between bacteria or bacterial products and their antisera. Each bacteriophage is a specific antigen independent from the bacterial antigens present in lytic

filtrates, and the antigenic properties are affected by the same influences that affect other known antigens, as for example heat. The reaction of neutralization seems to be analogous to the neutralization of toxin by antitoxin, in that complement is unnecessary, neutralization is not instantaneous but takes some time, and the reaction follows the law of multiple proportions. The fact that each phage is neutralized only by its specific antiserum makes possible the serological identification and classification of bacteriophages.

Antilytic sera may at times be of some value in the laboratory. A mixture of phages may be partially purified by using a serum which neutralizes one or more components, leaving the remainder active and thus simplifying the problem of their separation. Lysogenic cultures may be rendered free of phage by cultivation in broth containing serum active against the phage carried. The ensuing neutralization of phage permits the culture to return to its original state.

The use of the reaction of neutralization in the diagnosis of disease seems, in view of the results reported here, to be impractical. The failure of antibacterial sera to neutralize phage, and the indefinite number of phages acting on the same bacterial species would render the test uncertain. Furthermore, only positive results would be of any significance, since it would be difficult to exclude the presence of antibodies against some other bacteriophage acting on the same bacterium.

#### SUMMARY.

1. Each bacteriophage is a specific antigen.
2. The antibodies against the bacteriophage are independent of those against the bacterial substrate used in preparing the phage.
3. The reaction of neutralization is closely analogous to that of the neutralization of toxin by antitoxin.
4. The serum of mice experimentally infected with mouse typhoid (M.T.<sup>3</sup>) did not become capable of neutralizing phages acting on the infecting organism.
5. The use of the reaction of neutralization of bacteriophage in the diagnosis of disease as proposed by Sonnenschein seems impractical.



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## STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

### XII. CONCERNING THE PRODUCTION OF PHAGE FROM BACTERIAL CULTURES.

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The development of bacteriophage in bacterial cultures has been described repeatedly. The importance of the phenomenon is self-evident. If the production of phage from bacteria themselves can be incontrovertibly proved, then it is scarcely conceivable that the bacteriophage can be a living ultramicroscopic parasite of the bacteria.

In some cases, plaques from which lysis in series can be conducted have appeared spontaneously in stock cultures (1). In most cases, however, a more complicated procedure has been necessary (2) for the development of the phage, one involving repeated filtrations and reinoculations, sometimes of young cultures, but usually of cultures that have aged or been treated with some chemical or physical agent. The objection to all of the results is that they cannot be reproduced at will, and although positive results may predominate in the hands of some workers, the outcome of individual experiments is uncertain. Furthermore, the amount of manipulation necessary increases the possibility of contamination with phage, and this factor becomes of increasing importance when one considers the ubiquity of phage and its relative resistance to most chemical and physical agents. Consistent results were reported at one time by Putter and Vallen (3), but they subsequently traced them to the use of contaminated filters (4), and therefore retracted their previous statements. The possibility that bacteriophage was already present in the cultures used must also be considered. In this connection Manninger (5) takes the extreme view that all of the cultures of the colon-typhoid-paratyphoid group of bacteria are contaminated with bacteriophage.

A phenomenon distinct from these, however, and one which can be

regularly brought about is the production of phage for one bacterium in cultures of another.

This was first described in 1922 by Lisbonne and Carrère (6), and they considered it the result of bacterial antagonism. Kuttner (7), in 1923, produced phage from stock cultures, and the phage produced acted only on heterologous strains, and not on the strain from which it was derived. In 1924, Gildemeister and Herzberg (8) described a bacterium (*Coli* 88) having the property, like the *B. coli* strain of Lisbonne and Carrère, of initiating serial lysis of the Shiga bacillus. These two bacterial strains have similar, if not identical, properties, so the literature on the two will be summarized together.

Bordet was able, by culturing from single colonies, to secure strains that would not produce phage for the Shiga bacillus (9), and by subculturing single colonies for a number of passages, he could get strains that were to some extent lysogenic for each other (10). Since the great majority of the strains from single colonies could still produce phage for the Shiga bacillus, he called this phenomenon "active lysogenesis," as contrasted to "passive lysogenesis" which resulted from artificially mixing a culture with phage, and in which the phage was only perpetuated in mass cultures.

D'Herelle (11) repeated Bordet's experiments, and found that in artificially produced lysogenic cultures phage persisted in transfers from single colonies if the "rough" colonies were selected. He thought that such colonies indicated symbiosis of the bacteriophage with the bacteria. Bail (12) confirmed d'Herelle's experiments and agreed with him that the phenomenon did not represent a true production of phage from the bacteria themselves but resulted from the admixture of phage with the bacteria. McKinley (13) immunized animals with broth cultures of the *coli* strain of Lisbonne and Carrère and the resulting sera neutralized the phage already produced by the culture, but would not render the strain of bacteria non-lysogenic.

To explain the phenomenon of phage production for the Shiga bacillus in cultures of *B. coli* on the basis of phage preexisting in the culture, it is necessary to assume that the colon bacillus employed for the work carries a phage active against both *B. coli* and against *B. dysenteriae* Shiga, and that the particular strain of *B. coli* employed is resistant to this phage to such an extent that no gross lysis occurs, although enough individuals in the culture prove susceptible to serve for the perpetuation of the phage. It should be possible to reproduce the phenomenon experimentally, and this we have attempted to do in the following work. In addition to using a phage acting on both *B. coli* and *B. dysenteriae* Shiga, a monovalent phage, acting only on *B. coli*, was used. In the case of this latter the original strain of bacteria was necessary to demonstrate the presence of phage.

## EXPERIMENTAL.

*The Production of Lysogenic Bacteria.*—*B. coli*, and P.C. phage, which acts both on *B. coli* and on *B. dysenteriae* Shiga, were inoculated into a tube of broth, and incubation was continued after the completion of lysis. The resulting overgrowth was inoculated directly onto agar slants. The growth appeared normal, but phage was readily demonstrated by cultivating in broth either with *B. dysenteriae* Shiga or with the original strain of *B. coli*, then removing the living resistant bacteria by filtration, centrifugation, or heating to 56°C. for 40 minutes, and adding some

TABLE I.

*Serological Identification of Phage Recovered from Lysogenic Bacteria.*

Phage derived from:	Serum	Bacterium	Phage dilution			
			Undiluted	1-100	1-10,000	1-1,000,000
P.C. lysogenic <i>B. coli</i>	Normal	<i>B. coli</i>	+++	++	100±	—
	Anti P.C. phage	" "	—	—	—	—
	" B.W. "	" "	+++	++	100±	—
B.W. lysogenic <i>B. coli</i>	Normal	" "	+++	+++	26	1
	Anti P.C. phage	" "	+++	+++	23	1
	" B.W. "	" "	++	++	—	—

+++ = complete lysis.

++, + = lesser degrees of lysis.

Numbers indicate the number of plaques.

± following a number indicates that the number was estimated.

— indicates absence of lysis.

These signs will be used in the following protocols whenever lysis on agar slants is recorded.

of this material to a fresh culture of the susceptible organism. Phage could in this way be demonstrated even after the bacteria had been cultivated in series through 80 daily passages. A like experiment was done with B.W. coliphage, which acts only on *B. coli*. The phage could be demonstrated by allowing the resulting lysogenic strain to act on the original strain of colon bacilli.

*Identification of the Phages Produced by Lysogenic Bacteria.*—It was necessary to ascertain that the phages produced were those to which the bacteria were originally exposed. This was readily done, as the two phages are distinct serologically, and a serum prepared against one of the phages is inactive against the other.

Serial dilutions of the phages recovered were mixed with equal quantities of sera prepared against the original phages, and after an interval of 1 hour at room temperature, drops of the mixtures were run down the center of agar slants previously inoculated with the susceptible organism (Table I).

The phage recovered from the culture made lysogenic by exposure to P.C. phage was found to be neutralized by anti P.C. phage serum and not by anti B.W. phage serum, while the reverse was true of the phage recovered from the culture exposed to the action of B.W. phage. This establishes the identity of the phages recovered from the bacteria with those to which the cultures were originally exposed.

*Serological Detection of Phage in Lysogenic Cultures.*—It seemed reasonable to

TABLE II.

*Phage Neutralization by Sera Prepared against Lysogenic Bacteria.*

Phage	Serum	Bacterium	Phage dilution			
			Undiluted	1-100	1-10,000	1-1,000,000
P.C. coliphage	Normal	<i>B. coli</i>	+++	++	—	—
	Anti killed P.C. lysogenic	" "	—	—	—	—
	" live " "	" "	—	—	—	—
B.W. coliphage	Normal	" "	+++	++	60±	—
	Anti killed B.W. lyso- genic	" "	++	40±	1	—
	Anti live B.W. lysogenic	" "	—	—	—	—

assume that if phage is present in a culture, it should act as an antigen, and consequently, an animal immunized against a lysogenic culture should give a serum capable of neutralizing the phage carried. Animals were therefore immunized by intravenous injection, with the following antigens:

1. P.C. lysogenic *B. coli*, 18 hour broth culture.
2. P.C. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.
3. B.W. lysogenic *B. coli*, 18 hour broth culture.
4. B.W. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.

The sera of the immunized animals were then tested for ability to neutralize P.C. and B.W. coliphages, and the results are recorded in Table II. From this it is seen that the sera of animals immunized with lysogenic cultures are capable

of neutralizing the phage carried. It has been shown in a previous communication (14) that antibacterial sera are without effect on phage, so the ability of an antibacterial serum to neutralize bacteriophage must indicate that the bacteriophage was present in the culture used as antigen.

An attempt was then made to carry out analogous experiments with a culture of colon bacilli that was lysogenic when isolated. A culture of the *B. coli* strain of Lisbonne and Carrère was kindly furnished us by Dr. E. B. McKinley, and a rabbit was immunized by intravenous injections of 18 hour broth cultures, freshly prepared for each injection. After the course of immunization usually followed in this laboratory (3 daily injections at weekly intervals for 4 weeks) the serum was tested for ability to neutralize the phage secured by the action of this strain of bacteria on the Shiga bacillus.

TABLE III.

*Neutralization of Phage by Serum against B. coli Strain of Lisbonne and Carrère.*

Phage	Serum	Bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
Phage from <i>coli</i> strain of Lisbonne and Carrère	Normal Anti <i>B. coli</i> of Lisbonne and Carrère	Shiga L " "	10 <sup>-8</sup>	++	+	4	—
			10 <sup>-6</sup>	+	30±	—	—

Table III shows that, while the degree of neutralization was not great, there was definite neutralization of the phage by the immune serum.

This is considered definite evidence that the phage was present in the culture. The antilytic property of serum of animals immunized against this bacterium has also been reported by McKinley (13) and confirmed by da Costa Cruz (15).

The phenomenon, as experimentally reproduced, consists in the demonstration of the phage which was previously mixed with the bacterial culture, and which remains present through an indefinite number of transfers.

## DISCUSSION.

Consistent production of phage by one bacterium for another seems, in view of the experiments recorded here, to be the result of contamination of the "active" strain with a bacteriophage capable of acting on the susceptible strain. The majority of the individuals in the lysogenic strain are resistant and therefore such a culture cannot be used in the demonstration of the phage carried, unless, by picking a sufficiently large number of isolated colonies, a susceptible strain can be obtained. This has been done by Bordet, though his explanation does not agree with the one here given.

The phenomenon is readily reproducible experimentally by exposing a bacterial culture to the action of a polyvalent phage, the lysogenic strain thus obtained producing a phage that has the same range of activity as that possessed by the original phage. The apparent production of phage consists merely in the demonstration of the presence of the phage previously added, and that persists in the culture indefinitely. To demonstrate more conclusively that the phage recovered is the same one originally added to the culture, advantage can be taken of the fact that each phage is a specific antigen, and so the phage recovered can be identified with the original phage by its neutralization by specific antiserum.

Furthermore, the presence of phage in the bacterial culture may be detected by the immunization of animals, the resulting antibacterial serum being also capable of neutralizing the phage carried. As antibacterial sera are known to be incapable of neutralizing bacteriophage, the presence of neutralizing antibodies may be considered evidence that phage was present in the culture. The absence of neutralizing antibodies in an antibacterial serum, however, cannot be taken as positive evidence of the absence of bacteriophage, as antigens vary in their ability to stimulate antibody production. The possibility must be considered, therefore, that the phage carried may be a poor antigen and that no detectable antilytic properties may develop during the course of immunization. The development of antilytic properties during the immunization of animals with the *B. coli* strain of Lisbonne and Carrère shows definitely that phage was present in the culture.

The production of phage from bacterial cultures alone must be regarded as unproven, though this possibility must still be considered.

## SUMMARY.

1. The phenomenon of phage production by one bacterial culture for another of different sort has been reproduced experimentally.

2. This phenomenon results from phage carried with the culture, and not from the spontaneous appearance of phage in a culture previously free from it.

3. Animals immunized against the lysogenic bacteria may develop antibodies that neutralize the phage carried.

4. The development of neutralizing antibodies on immunization with a bacterial culture is evidence of the presence of bacteriophage in the culture.

5. The failure of such antibodies to appear on immunization with bacteria does not necessarily indicate that bacteriophage is not present.

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## ON THE INHERITANCE OF AGGLUTINOGENS OF HUMAN BLOOD DEMONSTRABLE BY IMMUNE AGGLUTININS.<sup>1</sup>

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Studies on the inheritance of serological properties were first undertaken systematically by von Dungern and Hirschfeld with the agglutinable substances in the blood of dogs (2) and with the human isoagglutinogens (3).<sup>2</sup> The authors named discovered the fact that the isoagglutinogens A and B are inherited as Mendelian dominants and this result has been amply confirmed by numerous workers.

According to their hypothesis there are two pairs of allelomorphous genes, *Aa*, and *Bb*, where *A* and *B*, the dominant genes, determine the presence of the corresponding agglutinogens, and *a* and *b*, the recessive genes, their absence. The genes for the blood groups are the following; group O:<sup>3</sup> *aabb*; group A: *AAbb* or *Aabb*; group B: *aaBB* or *aaBb*; group AB: *AABB* or *AABb* or *AaBB* or *AaBb*.

Another hypothesis has been advanced by Bernstein (6). He assumes multiple (three) allelomorphs, *R*, *A*, and *B*. The genetic formulæ accordingly are; group O: *RR*; group A: *AA*, *AR*; group B: *BB*, *BR*; group AB: *AB*. The theory of Bernstein does not involve a deviation from the older theory in the types of offspring except in the cases of parents belonging to group AB. According to the older view there may be children of any group in unions where one or both of the parents are in group AB; Bernstein's hypothesis, on the other hand, excludes children of groups O and AB in unions *O* × *AB*, and children O in unions *A* × *AB*, *B* × *AB*, or *AB* × *AB*. The recent work especially of Schiff (7), Thomsen (8), Preger (9), and Sievers (10) supports the opinion of Bernstein.<sup>4, 5</sup>

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<sup>1</sup> See the preliminary report (1).

<sup>2</sup> The problem of the inheritance of the human blood groups and a few results had been mentioned by Ottenberg and Epstein (4).

<sup>3</sup> The nomenclature of the blood groups by letters instead of numerals has been recommended both by the American Association of Immunologists and by the National Research Council (5) and is used in the present publication.

<sup>4</sup> The objection of Mendes-Correa (11) to the theory of Bernstein would imply

TAB  
*Tests for M and N in Several Fam*

Family No . . . . .	143						144					
Blood No. ....	F 298	M 299	300	301	302	303	F 304	M 305	306	307	308	309
Group. ....	A	A	A	A	A	A	O	O	O	O	O	O
Reaction for M. ....	+++	+++	++±	++±	+++	+++	++±	++±	++±	++±	+++	++±
Reaction for N. ....	-	-	-	-	-	-	±±	±±	±±	±±	-	±±

The strength of the reactions is indicated by the signs +, +±, ++, ++±, +++.

TABLE II.  
*Heredity of the Agglutinin M.*

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		M+	M-	M+	M-
M+ × M+	101	403	33	92.4	7.6
M+ × M-	59	165	85	66.0	34.0
M- × M-	6	0	29	0	100

## I.

F = Father; M = Mother.

145					146						147					
M 311	312	313	314	315	F 316	M 317	318	319	320	321	F 322	M 323	324	325	326	327
A	A	A	A	A	O	A	O	A	A	O	A	O	A	A	O	A
++	++	++±	-	++±	++	++±	++±	++±	++±	++±	-	++±	-	++±	-	-
±	+	+	++	±	-	++	±	±	-	-	++±	++	++±	±	++±	++

TABLE III.

*Heredity of the Agglutinin N.*

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		N+	N-	N+	N-
N+ × N+	31	130	18	88.5	11.5
N+ × N-	29	81	40	66.9	33.1
N- × N-	4	0	17	0	100

*F = Father; M = Mother.*

145					146						147					
M 311	312	313	314	315	F 316	M 317	318	319	320	321	F 322	M 323	324	325	326	327
A	A	A	A	A	O	A	O	A	A	O	A	O	A	A	O	A
++	++	++	-	++	++	++	++	++	++	++	-	++	-	++	-	-
+	+	+	++	+	-	++	+	+	-	-	++	++	++	+	++	++

TABLE III.

*Heredity of the Agglutinin N.*

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		N+	N-	N+	N-
N+ × N+	31	130	18	88.5	11.5
N+ × N-	29	81	40	66.9	33.1
N- × N-	4	0	17	0	100

The investigations outlined on the heredity of human blood groups are not only of theoretical interest<sup>6</sup> but they have attracted much attention because of their practical application in forensic medicine. A certain limitation lies in the fact that only two properties could be utilized. It is true that some experiments pointed to the existence of differences in human blood aside from the blood groups (13-16), but as a result of these studies no genetic investigations worthy of notice have been reported although obviously such would have been desirable. The reason for this is to be seen in the lack of workable methods.

The observations reported in previous publications (1, 17, 18) enabled us to undertake a study of the heredity of serological properties of human blood other than those determining the four blood groups.

With regard to the property designated as M there was no difficulty in selecting immune sera and absorbing bloods in such a manner that the reactions were either entirely negative on microscopic examination or so strong that clumps were visible to the naked eye.

The results with a second property, N, whose heredity was studied, varied depending upon the particular immune serum used. Although the strongest agglutinations occurred with the same bloods, there were differences in the reactions of minor strength so that the number of positive tests was greater with some sera than with others. In the following experiments two sera were selected which behaved identically and gave the smallest number of positive reactions; *i. e.*, the bloods acted upon by these sera reacted positively with all sera. Moreover with the sera chosen there was a distinct break between positive and negative tests, a point of significance for the present issue.

The frequency of the types M+ and M-, and N+ and N-, as already stated, is approximately the same in the four blood groups. According to our present results, there were in 1708 white individuals 326 (19.1 per cent) with negative reactions for M, and in 532 white individuals there were 139 (26.1 per cent) negative for N.

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that the formula  $p + q + r = 1$ , holds for arbitrarily chosen values, which is obviously not the case.

<sup>6</sup> While this paper was in press, another explanation based upon the assumption of incomplete linkage was proposed by Bauer (*Klin. Woch.*, 1928, vii, 1588).

<sup>6</sup> Cf. Morgan (12).

The technique of performing the tests has been described (18). The absorptions and tests for N were made at about 40°C.

It should be stated that the technique offers some difficulties as compared to the common isoagglutination tests. It is necessary to become well acquainted with the method and to know the properties of each serum in order to absorb completely all agglutinins but those in question.

The material for this study was obtained from two maternity clinics in the City of New York. Altogether 166 families were studied; in most of these (122) there were four or more children. Several families were always included in one experiment and also a number of control bloods with known properties.

TABLE IV.

Unions No.	Type of parents	No. of families	No. of children of types		
			M+N+	M+N-	M-N+
1	M+N+ × M+N+	11	31	17	7
2	M+N+ × M-N+	17	40	1	34
3	M+N+ × M+N-	24	60	40	3
4	M+N- × M-N+	5	17	0	1
5	M+N- × M+N-	4	0	17	0
6	M-N+ × M-N+	3 (6)	0	0	18 (29)

The figures in parentheses in unions of type 6, include the three families tested only for M but which, according to our experience, would be of the type M-N+.

In 166 families only the property M was investigated; 64 families were examined for M and N. A representative experiment is given in Table I.

The results for M are summarized in Table II and are arranged in three classes corresponding to the three types of unions and those for N in Table III are similarly arranged.

64 families were examined both for M and N. The results (Table IV) are arranged according to the six sorts of matings and the three types of offspring that have been observed.

A list of the tests is given in Tables Va and Vb for the individual families, the former showing the tests for M (102 families) and the latter tests for both M and N (64 families). In each case the children are

TABLE Va.\*\*  
Reactions for M.

Family No.	Father	Mother	Children				
1	O+	B+	O+ ♂	O+ ♂	O+ ♀	O+ ♀	
2	O+	O+	O- ♀	O- ♂	O+ ♂		
3	AB-	AB+	AB- ♀	B+ ♂	B- ♂	A- ♀	
4	A+	A+	A+ ♂ A+ ♂	A+ ♀	A+ ♀	A- ♂	A- ♂
5	A+	A+	A+ ♀	A+ ♂	O+ ♀	A+ ♂	
6	B-	A+	B+ ♀	B+ ♀	B+ ♂	B+ ♂	
7	O+	O+	O+ ♂	O+ ♀	O+ ♂	O+ ♂	
8	AB+	A+	A+ ♂	B+ ♀	A+ ♂	A+ ♂	
9	A+	O-	A+ ♂ O+ ♀	O+ ♀	A+ ♀	O+ ♂	A+ ♀
10	B+	B+	O- ♂	B- ♀	B+ ♂		
11	O+	AB+	A- ♀	A+ ♂			
12	O+	A+	A+ ♀	O+ ♀	A+ ♀	A+ ♂	
13	O-	O-	O- ♀	O- ♀	O- ♀	O- ♀	
14	AB+	A+	A+ ♂	A+ ♂	A+ ♀	AB+ ♂	A+ ♂
15	AB+	A+	A+ ♀	AB+ ♀	AB+ ♂		
16	B+	O-	B- ♀	O- ♂	O+ ♀	O+ ♂	O- ♂
17	A+	O+	A+ ♂	O+ ♀	A+ ♂	O+ ♂	
18	A+	O+	O+ ♀	A+ ♀	A+ ♂*	A+ ♀*	O+ ♀
19	A+	O-	O+ ♀	O+ ♀	A+ ♀	O+ ♀	
20	A-	B+	O+ ♀	AB+ ♀	AB+ ♀	A- ♀	

\* Twins.

\*\* 20 of the 166 families examined were negro families.



TABLE Va—Continued.

Family No.	Father	Mother	Children				
21	A+	O+	A+ ♀	A+ ♀	O+ ♀	O+ ♂	
22	O+	O+	O+ ♀	O+ ♂			
23	O+	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
24	O+	O-	O+ ♂ O+ ♀	O- ♂ O+ ♀	O- ♂	O+ ♀	O+ ♂
25	B+	A-	AB- ♀ B+ ♀	B+ ♀	B+ ♂	AB+ ♀	AB+ ♂
26	AB+	A+	AB- ♂	A+ ♀	AB+ ♂	B+ ♀	B+ ♂
27	O-	A+	O- ♂	A+ ♀	A- ♂	A- ♂	
28	B+	O+	B+ ♂	O+ ♂	B+ ♀	O+ ♂	
29	O+	A-	A+ ♂	O- ♀	O- ♀	A+ ♀	O- ♀
30	O-	O+	O+ ♀	O- ♀			
31	A+	O-	A- ♀	O- ♀	A+ ♀	A+ ♀	
32	A+	O-	A- ♀	A+ ♀	A+ ♂		
33	O-	O+	O- ♂	O+ ♀	O- ♀	O- ♀	
34	B+	A+	A+ ♂	AB+ ♂	AB+ ♀	AB+ ♀	
35	A-	B+	A- ♀	AB- ♂	B- ♂	AB+ ♂	
36	O-	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
37	O+	A+	A+ ♀	A+ ♂	O+ ♀		
38	A+	A+	A+ ♂	O+ ♂	A+ ♂	O+ ♂	
39	O+	A-	A+ ♀	O+ ♂	O+ ♂		
40	O+	A+	A+ ♀	A+ ♀	A+ ♂	A+ ♂	A+ ♀
41	A+	B+	AB+ ♀ AB+ ♂	AB+ ♂	A+ ♀	AB+ ♀	AB+ ♂

TABLE Va—Continued.

Family No.	Father	Mother	Children				
42	A+	B-	A+ ♂	A- ♀	AB+ ♀	A- ♂	
43	A+	B+	A+ ♀	A+ ♂	AB+ ♀	A+ ♀	
44	B+	A+	A+ ♀	A+ ♂	A+ ♀		
45	B+	O-	O- ♂	B+ ♂			
46	AB-	O+	B+ ♀	B+ ♀*	B+ ♀*	B+ ♀	
47	A+	O+	O+ ♂	A+ ♀	O+ ♂	A+ ♂	O+ ♂
48	A+	A+	O+ ♂	O- ♂	O- ♂		
49	A+	A+	A+ ♀	A+ ♀	O- ♀	A- ♀	
50	B+	A+	B- ♀	O+ ♂	AB+ ♀		
51	O+	O-	O+ ♀	O+ ♀*	O+ ♀*	O+ ♀	
52	O+	O-	O+ ♂	O- ♀	O+ ♂	O- ♂	O- ♂
53	A+	B-	A+ ♀	AB+ ♂	AB- ♂	A- ♂	
54	A+	A+	A+ ♀	A+ ♂	A+ ♀		
55	A+	A+	A+ ♂	A+ ♀			
56	O+	A+	O+ ♂	A+ ♀	O+ ♀		
57	O-	O+	O+ ♀	O+ ♂	O+ ♀	O+ ♂	
58	O+	A+	A+ ♀	A+ ♂	A+ ♀		
59	O+	A+	O+ ♂	O- ♂	O+ ♂	O+ ♀	A+ ♂
60	B-	O-	O- ♂	O- ♂	O- ♀		
61	O+	A-	O+ ♀	O+ ♂	A+ ♀		
62	A+	A+	A+ ♀	O+ ♂	A+ ♂	O+ ♀	
63	O+	A+	O+ ♀	O+ ♂	A+ ♂	A+ ♂	A- ♂

\* Twins.

TABLE Va—Continued.

Family No.	Father	Mother	Children				
64	O+	O+	O+ ♂ O+ ♂	O+ ♂	O+ ♀	O+ ♀	O+ ♂
65	O+	O—	O+ ♂	O+ ♀			
66	O+	B+	B+ ♂	B+ ♂			
67	A+	O+	A+ ♂	O+ ♂			
68	O+	O+	O+ ♀	O— ♂	O+ ♀	O— ♀	
69	A+	O+	O+ ♀	O+ ♀	A+ ♀	O— ♀	A+ ♂
70	A+	O+	O+ ♀ A+ ♂	A+ ♂ O+ ♂	O+ ♀ O+ ♂	O+ ♀ A+ ♀	O+ ♀
71	AB—	O+	O+ ♀ O+ ♀	A+ ♀	B+ ♂	A+ ♂	A+ ♀
72	B—	A—	O+ ♀	O+ ♀	B— ♀	AB+ ♀	
73	A+	O+	O+ ♀	O+ ♀	A— ♂	O+ ♂	
74	O+	A+	A+ ♀	A+ ♂	O+ ♂	O+ ♂	
75	B+	O+	O+ ♂	O+ ♂	B+ ♂	O+ ♀	
76	O+	A+	O+ ♂ O+ ♂	O+ ♂	A+ ♀	A+ ♂	A— ♀
77	O—	B+	O+ ♂ O+ ♀	O+ ♀	O+ ♂	B+ ♀	O— ♂
78	O+	O+	O+ ♀ O+ ♀	O+ ♂ O+ ♀	O+ ♂ O+ ♂	O+ ♀	O— ♀
79	O+	B+	B+ ♂	B+ ♂	B+ ♂		
80	O+	B+	O+ ♂	B+ ♀	O+ ♂	B+ ♂	O+ ♂
81	O—	B+	O+ ♀	O+ ♂	B+ ♂		
82	O+	O+	O+ ♂	O+ ♂	O+ ♂		

TABLE Va—*Concluded.*

Family No.	Father	Mother	Children				
83	O+	O+	O+ ♂	O- ♀	O+ ♀		
84	O+	O+	O- ♀	O- ♂	O+ ♂	O+ ♂	
85	O+	B-	B- ♂	B+ ♂	B- ♂		
86	A+	B+	B+ ♀	O+ ♀	AB+ ♀		
87	O+	A+	O+ ♀ O+ ♀	A+ ♀	O+ ♂	O- ♀	A+ ♂
88	O+	O-	O+ ♂	O+ ♀	O+ ♂		
89	A+	O-	A+ ♂ O+ ♀	O+ ♂	O+ ♂	A+ ♂	O+ ♂
90	AB-	O+	A- ♂	B- ♀	A+ ♂		
91	O-	AB+	A- ♂ B- ♀	B- ♀	A- ♀	B- ♀	A+ ♀
92	O+	O+	O+ ♀	O+ ♂	O+ ♂	O+ ♂	
93	B+	B+	B+ ♂	B+ ♂	B+ ♂	B+ ♂	
94	O+	A-	A+ ♀	A- ♂	A+ ♂	A- ♂	A+ ♀
95	B+	A+	B+ ♀	B+ ♂	O+ ♂	A+ ♀	
96	A+	A-	O- ♂ O- ♂	A- ♀	A+ ♂	A+ ♀	O- ♂
97	A+	O+	A+ ♂ O+ ♂	A+ ♀	O+ ♀	O+ ♀	O+ ♂
98	O+	A+	O+ ♀	O+ ♀	O+ ♀	O+ ♂	O+ ♀
99	B+	O+	B+ ♂ O+ ♂	B+ ♀	O+ ♀	O+ ♀	O+ ♂
100	A+	A+	A+ ♂	A+ ♂	A+ ♂	A+ ♀	
101	B+	O+	O+ ♂ B+ ♀	B+ ♂ O+ ♀	B+ ♀	O+ ♂	O+ ♂
102	B-	A-	A- ♀	A- ♀	A- ♂	AB- ♀	

TABLE Vb.  
*Reactions for M and N.*

Family No.	Father	Mother	Children			
103	O++	A++	A++ ♂ A+- ♂	O+- ♀	A-+ ♂	O++ ♂
104	O-+	A++	O-+ ♀	O++ ♂	O++ ♂	A-+ ♂
105	O-+	O-+	O-+ ♀	O-+ ♂	O-+ ♂	O-+ ♂
106	O++	AB+-	B++ ♂	B++ ♀		
107	O+-	A++	O+- ♀	A++ ♂	A+- ♀	A+- ♀
108	O+-	A+-	O+- ♀	A+- ♀	O+- ♂	O+- ♂
109	O+-	A++	A++ ♂ O-+ ♂	O++ ♂	A+- ♂	A+- ♂
110	A++	O-+	A++ ♀	O++ ♀	O++ ♂	O++ ♀
111	A+-	A-+	A++ ♀ A++ ♂	A++ ♂	A++ ♀	A++ ♂
112	O++	O+-	O++ ♂ O++ ♂	O++ ♀	O++ ♀	O+- ♂
113	A-+	A++	A++ ♂	A++ ♀	A-+ ♀	
114	A++	O++	A-+ ♀ O+- ♂	O+- ♂ A++ ♂	O-+ ♀	A++ ♀
115	A+-	A++	A++ ♂	A++ ♀	A++ ♀	A++ ♂
116	O++	B-+	B-+ ♀ O++ ♂	O-+ ♀ B++ ♀	B-+ ♂	O-+ ♂
117	O-+	O++	O++ ♀ O++ ♂	O-+ ♀ O++ ♂	O-+ ♀	O++ ♂
118	O+-	A++	O++ ♀ A+- ♀ A++ ♀	O++ ♂ A++ ♀	O++ ♂ A+- ♀	O+- ♀ O++ ♀
119	O-+	A++	A-+ ♂ A++ ♂	A-+ ♀ A++ ♀	A++ ♀ O++ ♀	O-+ ♀

TABLE Vb—Continued.

Family No.	Father	Mother	Children			
120	A++	O-+	A++ ♂	A++ ♀	A++ ♂	A++ ♀
121	B++	A++	AB-+ ♂ A+- ♂	A++ ♂ A++ ♀	A++ ♀	A++ ♂
122	O+-	A++	O++ ♂ A++ ♂	A+- ♂	A+- ♂	A++ ♀
123	O-+	A+-	A++ ♂	A++ ♀		
124	A+-	A++	A++ ♂ A+- ♀	O+- ♀	A++ ♀	O+- ♂
125	O++	A++	A+- ♂ O++ ♀	A++ ♀	O++ ♀	A++ ♂
126	B++	B++	B++ ♀	B+- ♂	O++ ♂	
127	A+-	B++	B-+ ♂	A+- ♀	O+- ♂	
128	O+-	O++	O+- ♀	O++ ♀	O+- ♀	O++ ♂
129	O++	O+-	O+- ♂ O+- ♀	O+- ♂ O++ ♂	O++ ♀	O++ ♀
130	A-+	A-+	A-+ ♂ A-+ ♂	O-+ ♂ O-+ ♂	A-+ ♀	A-+ ♀
131	A+-	O-+	O++ ♀	O-+ ♀	O++ ♂	O++ ♂
132	A-+	O++	A++ ♀ O++ ♂	O-+ ♀	O-+ ♂	O++ ♂
133	O++	O+-	O+- ♂ O+- ♀	O++ ♀	O+- ♂	O+- ♀
134	O+-	A+-	A+- ♀	A+- ♀	A+- ♂	
135	O+-	O++	O++ ♀	O+- ♂	O++ ♀	
136	A++	A-+	A-+ ♂ O-+ ♀	A-+ ♂	A-+ ♀	A-+ ♀
137	B+-	O++	B++ ♂	B+- ♀		

TABLE Vb—Continued.

Family No.	Father	Mother	Children			
138	A+-	O++	O++ ♀	A+- ♀		
139	A+-	B-+	B++ ♀	B++ ♀	B++ ♀	AB++ ♀
140	A++	B++	O++ ♀ O++ ♀	O++ ♂ O++ ♂	A++ ♂	AB+- ♂
141	O++	B++	B++ ♀ O++ ♂	B++ ♂	O++ ♀	O-+ ♂
142	O+-	O++	O++ ♂ O-+ ♂	O++ ♂	O++ ♀	O++ ♂
143	A+-	A+-	A+- ♂	A+- ♀	A+- ♂	A+- ♂
144	O++	O++	O++ ♀	O++ ♀	O+- ♀	O++ ♂
145	O-+	A++	A++ ♂	A++ ♂	A-+ ♀	A++ ♀
146	O+-	A++	O++ ♀	A++ ♂	A+- ♂	O+- ♂
147	A-+	O++	A-+ ♀	A++ ♂	O-+ ♀	A-+ ♂
148	A++	B-+	B-+ ♀	A++ ♂		
149	O-+	B-+	B-+ ♂ O-+ ♂	O-+ ♀ B-+ ♂	O-+ ♀ O-+ ♂	B-+ ♂ B-+ ♀
150	O-+	A++	A-+ ♀	A++ ♀	A+- ♂	A-+ ♀
151	B+-	O-+	O++ ♀	B++ ♀	B++ ♀	
152	O-+	O++	O-+ ♂	O++ ♂		
153	A-+	O++	A++ ♂ O++ ♀	A-+ ♀ O-+ ♀	O-+ ♂	O-+ ♂
154	A++	B+-	O++ ♀	B++ ♀	O+- ♂	
155	A+-	A++	A++ ♂ A+- ♂	A+- ♂ A++ ♂	A++ ♂	A+- ♂
156	A++	O++	O++ ♀	O+- ♀	O+- ♂	A+- ♀

TABLE Vb—*Concluded.*

Family No.	Father	Mother	Children			
157	O—+	O++	O++ ♀ O++ ♀	O++ ♂ O—+ ♂	O—+ ♀	O—+ ♀
158	O++	A—+	A++ ♀	A++ ♂	O++ ♂	
159	O++	B+-	B++ ♀	O++ ♀	B++ ♀	B++ ♂
160	A++	O++	A+- ♂ O++ ♂	A++ ♀ O+- ♀	O+- ♂	A—+ ♂
161	O++	A++	A++ ♀ A—+ ♀	A++ ♀	A+- ♀	A+- ♂
162	A+-	O++	A++ ♂ O++ ♀	A++ ♂	A+- ♀	A+- ♂
163	B+-	O+-	O+- ♂ O+- ♂	B+- ♂ B+- ♂	O+- ♂	B+- ♂
164	B+-	A++	O++ ♀ AB++ ♂	A++ ♂	A++ ♂	O++ ♂
165	B+-	O++	O++ ♀	O+- ♀	B+- ♀	O+- ♀
166	B+-	O++	B++ ♀	O++ ♀	B+- ♂	

recorded in order of decreasing age beginning with the eldest. The letters designate the groups, and the signs + and - the reactions for M (Table Va). In Table Vb the first + or - sign designates the test for M and the second sign that for N.

As to the heredity of the factors A and B our results agree with the established fact that they are inherited as Mendelian dominants, except for three families in which A or B appeared in children when they were absent in the blood of the parents. These cases were considered as instances of illegitimacy and were excluded from the tabulations. One of these families was examined only for M and two for both M and N. The results were:

Father	Mother	Children			
O+	O+	O+ ♂	B+ ♂	O+ ♀	A+ ♀
O++	O++	A++ ♀	A++ ♀	O++ ♀	O+- ♀
O+-	B—+	AB++ ♀	O++ ♂	O++ ♀	O++ ♀



In family 71,  $AB \times O$ , there were two children in group O; the mother refused reexamination.

From the data reported it is evident that the agglutinogens studied are inherited properties. If we consider M and N separately they would seem to behave like Mendelian dominants. The characters cannot be recessive since in unions  $+ \times +$  there are children whose blood lacks the property. This result is to be expected if there are individuals among the parents heterozygous for a dominant character. If the absence of the agglutinogens is recessive there should occur no positive reactions in children from unions  $- \times -$ . This is actually borne out by the observations. Thus in six such families with M- parents all the children (29 in number) gave negative reactions for M; likewise in the four families with N- parents all the children (17 in number) belonged to the N- type. In this respect our findings are analogous to the rule established by von Dungern and Hirschfeld for the isoagglutinogens A and B, *i. e.*, that these do not appear in the offspring if they are absent in both parents.

In order to discuss the numerical results for M alone in the three sorts of matings it is necessary to know the incidence of homozygous (MM) and heterozygous (Mm) individuals among the M+ parents. From a formula quoted by Johannsen (19), the following values are obtained:  $MM = 29.6$ ;  $Mm = 49.6$ ;  $mm = 20.8$  (approximately 30, 50, 20, respectively). According to this formula the percentage of homozygous individuals equals  $100 - 20 \sqrt{R} + R$ , that of heterozygous  $20 \sqrt{R} - 2R$ ; where  $R$  is the percentage of recessive (M-) individuals observed in the population. The figures of M+ and M- reactions are taken from all individuals, including the parents, of the 166 families examined for M.

Calculating from these figures the number of M- children in the unions  $M+ \times M+$  one has to consider only those in which both parents are heterozygous, *i. e.*, approximately  $5/8 \times 5/8$ ; since  $1/4$  of the children of these matings should be M-, 9.8 per cent of M- children are to be expected; the observed value is 7.6 per cent.

In the unions  $M+ \times M-$  50 per cent of the offspring of heterozygous M+ parents may be expected to be M-; *i. e.*,  $5/8 \times 1/2 = 31.3$  per cent; actually 34 per cent M- children were found.

In the smaller series where both factors were examined (see Table IV) the agreement between the calculated and the observed values is not so satisfactory.

Applying the formula of Johannsen for the factor N we obtain  $NN = 23.1$ ;  $Nn = 49.8$ ;  $nn = 27.1$ . Calculated as above the figures are in matings  $N+ \times N+$  11.7 per cent  $N-$  children (observed 11.5 per cent) and in matings  $N+ \times N-$  34.2 per cent  $N-$  children (observed 33.1 per cent).

So far the cursory analysis of the results does not contradict the idea of two independent factors. However, there is evidence which does not seem compatible with this view. In the first place, if the factors were independent one would expect a certain, although small, percentage of bloods to lack both M and N, that is, if the genotype  $M-N-$  is not lethal, or its phenotype indistinguishable from one of the other types. In fact, as has been stated formerly (18) no such case has been found in the examination of more than 1200 specimens<sup>7</sup> and in each  $M-$  blood the reaction for N was found to be very strong. Further evidence emerges from an analysis of results in families examined for both properties. One sees that the frequency of  $M-$  children in the three sorts of matings, Nos. 1, 3, and 5 (Table IV) of parents  $M+$ , varies greatly according to the N reactions of the parents and that likewise the occurrence of children  $N-$  in matings 1, 2, and 6 is influenced by the presence or absence of M in the parents. A similar statement holds for the appearance of  $M-$  or  $N-$  children in matings 2 and 4, and 3 and 4, respectively.

Actually in most of the six sorts of unions the observed figures do not tally satisfactorily with those to be expected on the basis of two independent factors if one computes the expectancy from the figures given above for heterozygous and homozygous individuals. Thus in matings 2 and 3 there are too many children of the type  $M-N+$  or  $M+N-$ , respectively, and in union 4 there appear with one exception only children of type  $M+N+$ . These numerical results could be interpreted in various ways. One hypothesis consists in assuming two genes which, when homozygous, would determine the phenotype  $M+N-$  and  $M-N+$  respectively, while the phenotype  $M+N+$

<sup>7</sup> This number includes the blood of negroes and Indians. About 900 of these were tested with the improved technique, namely, performing the tests for N at about 37-40°C.

would correspond to the heterozygous gene constitution. This view accounts for the non-existence of the type  $M-N-$ . On the basis of this hypothesis the expected values for the types of offspring are: mating 1:  $M+N+$  50 per cent,  $M+N-$  25 per cent,  $M-N+$  25 per cent; mating 2:  $M+N+$  50 per cent,  $M-N+$  50 per cent; mating 3:  $M+N+$  50 per cent,  $M+N-$  50 per cent; mating 4:  $M+N+$  100 per cent; mating 5:  $M+N-$  100 per cent; mating 6:  $M-N+$  100 per cent.<sup>8</sup> Allowing for the relatively small number of individuals examined these figures agree fairly with those observed and better than the figures calculated for independent factors. Especially striking is the fact that in matings 2 and 3 there are almost no children  $M+N-$  or  $M-N+$ , respectively, and in mating 4 only one child not of the type  $M+N+$ . Still, there are five cases which contradict the hypothesis mentioned, namely, the individual  $M+N-$  in union 2, the three children  $M-N+$  in union 3, and one child  $M-N+$  in union 4. To attribute all these five exceptions to illegitimacy seems hazardous since only a proportion of illegitimate children would be detected by the tests employed and because the number of the exceptional cases is high compared with that of children which do not conform to the rule of von Dungern and Hirschfeld.

It may be pointed out that in all of the five exceptional cases in unions 2, 3, and 4, the father and not the mother is of the type opposite to that of the child, *e. g.*, father  $+-$ , child  $-+$ .

On the basis of the assumption just discussed and with the aid of the formula used above, the frequency of one type could be used to calculate the frequency of the other two types. Starting from the figure 20.8 for  $M-$  in a certain population the computation gave the value of 29.6  $N-$ ; similarly in a population of 205 Indians examined by us, the observed value of 4.9  $M-$  leads to an expectancy of 60.7 for  $N-$ . Both these figures are in good agreement with those observed, namely, 26.1 and 60.0 respectively.<sup>9</sup>

An alternative hypothesis would suppose a close linkage between  $M$  and  $N$ . If, then, the gene combinations  $Mn$  and  $mN$  are numerically predominant this could explain the observed figures and also the occurrence of the exceptional cases aforementioned, but unless one assumes a lethal effect there arises a difficulty from the following con-

<sup>8</sup> The numbers of the matings refer to those given in Table IV.

<sup>9</sup> These results will be discussed in another publication (20).

sideration. If the factors M and N have been in the race for a long time the occurrence of cross-overs should by now have reduced the assumed numerical difference. However, on account of the existence of agglutinogens similar to M and N in anthropoids (chimpanzees) (18) it does not seem likely that they are due to recent mutations.

If in the unions listed as No. 1, Table IV, one of the parents be homozygous with respect to both M and N, then all children of such a union would be of the phenotype  $M+N+$ . The fact that this was not the case in any of the eleven families shows that none of the parents can be homozygous for both M and N, but such homozygous parents may have occurred in unions 2 and 3.

In view of the limited number of families studied it would seem premature to attempt a final interpretation and to discuss further possibilities such as the existence of more than two allelomorphs. Also it has to be considered that the state of affairs may be complicated, *e. g.*, by interacting or modifying effects of factors determining hitherto unknown agglutinable structures.

It may be added that there is no indication of a linkage between M and N and the isoagglutinogens A and B, and, as in the case of A and B, no evidence of a sex linkage.

#### SUMMARY.

The heredity of two agglutinable structures demonstrable by immune agglutinins was studied in 166 families. From the data collected it is evident that one deals with a case of Mendelian inheritance. The main result of the studies is the demonstration that it is feasible to investigate the heredity of serological structures of human blood other than the group agglutinogens. Irrespective of the ultimate theory it seems very probable that the properties M and N do not appear in the offspring when they are absent in both parents—a conclusion substantiated by the examination of ten families with 46 children. These findings offer the prospect of forensic application to cases of disputed paternity and, in our opinion, a correct decision could already be given, at least with great probability, provided the reagents are available and the method properly applied. Of course further work is needed before the test can be adopted as a routine procedure.

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## CELL ANTIGENS AND INDIVIDUAL SPECIFICITY.<sup>1</sup>

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The general aspects of immunological specificity have been discussed before this society in a masterly manner by authorities like Hektoen and Wells, on former similar occasions. If the following review concerns itself again with related matters, this may be justified by the broadening of the subject of serological specificity which has branched off from medical immunology as a somewhat independent line of research.

The interest in the particular topic of blood antigens has been revived of late by investigations centering round several distinct issues, such as the principles of species specificity, the chemical nature of cell antigens and the human blood groups. Beginning with the blood groups, one of the questions which has arisen is whether the differences of human blood are limited to the four groups or whether there are other variations. For cattle blood a great individual variety with regard to agglutinogens has been established by Todd and White (1) with the use of isohemolysins of sera prepared for the prevention of cattle plague.<sup>2</sup> The view that a similar condition obtains in human blood, though kept in mind since the first studies on isoagglutinins, lacked satisfactory evidence or at least was not based upon readily reproducible experiments. Accordingly, in recent comprehensive reviews of the subject (Lattes, Hirsfeld (5)) little consideration is given to the question. This state of things is of but minor consequence for practical purposes since the main application of blood grouping, namely for transfusion, has, broadly speaking, reached a satisfactory stage. The issue however has a bearing upon the general problem of biochemical individuality.

As far as isoagglutinins were employed there are only few reliable

<sup>1</sup> Presidential address delivered to the American Association of Immunologists, April 30, 1928, Washington.

<sup>2</sup> Cf. the results with the blood of goats, dogs, chickens (2, 3, 4).

data on marked exceptions to the group rule. Some very suggestive results were reported by v. Dungern and Hirschfeld (6) who performed agglutinin tests with human blood and absorbed normal animal sera. These investigations were not carried further owing to the difficulty in securing consistent and unequivocal results which would permit of a systematic study of the agglutinable elements concerned and their distribution. A similar remark applies to another method, namely the agglutination of human blood by human sera at low temperature. Although this type of agglutination also takes place with the serum and blood of the same person it was possible to find individual variations by means of this method (7, 8, 9). These differences vary quantitatively and the tests are not strongly positive or negative like common isoagglutination as one would expect from some of the reports published.

Clear-cut results were secured recently (10) with the use of hemagglutinating sera from rabbits injected with human blood. After exhaustion of the sera with particular blood specimens, solutions were obtained that differentiated human bloods. A certain difficulty of this technique consists in the preparation of some of the necessary immune sera. The long known fact that the response to antigens varies greatly with particular animals is more than usually pronounced in this instance and good sera are formed by a few animals only. But, once the sera are available, the reactions are as a rule very marked and leave no doubt as to the existence of differences within the groups. The observations agree with the idea that there are a number of more or less definite agglutinable factors each of them present in many but not in all blood specimens and occurring with approximately equal frequency in the four blood groups. Hitherto at least three such elements have been found and while it is not possible to determine the total number of such structures we are of course unable to assert that we have succeeded in finding all or even most of them. Additional agglutinable factors which do not correspond to those just discussed could be demonstrated by an abnormal isoagglutinin described by Ottenberg and Johnson, and by another, observed in our laboratory in the serum of an individual after transfusion with blood of the same group. Since every new character would about double the number of blood types and taking into account quantitative grada-



tions, one may surmise that almost every blood has peculiar serological features.<sup>3</sup> That is what one would anticipate if the serological diversity is of the same sort as that observed in transplantation experiments, considering that transplantations as a rule succeed only with tissues from the same individual.

The nature of what was referred to as serological structures is still uncertain. One may assume that the reactions are due either to the presence of a multitude of substances in a single cell or more likely to variations in the molecule or complex forming the antigen. That the isoagglutination properties A and B are independent of a morphological structure or a particular arrangement of the cell constituents follows from the demonstration of group specific reactions with alcoholic blood extracts (11). For the new characters analogous investigations are still required. At any rate these must be regarded as biological entities and as constitutionally determined, for it was possible to show that at least two of them are in all probability instances of Mendelian heredity (12) like the isoagglutinogens A and B. In examining families for one of the properties (M) the following percentages were found: In 101 matings  $+$   $\times$   $+$ , 7.6 per cent children with negatively reacting blood; in 59 matings  $+$   $\times$   $-$ , 34 per cent; in 6 matings  $-$   $\times$   $-$ , 100 per cent (29 children). For a second property (N) the figures were: 31  $+$   $\times$   $+$ , 11.5 per cent; 29  $+$   $\times$   $-$ , 33.1 per cent; 4  $-$   $\times$   $-$ , 100 per cent (17 children). Thus no positive reactions were found in the offspring of  $-$   $\times$   $-$  matings where both parents are supposed to be recessives and in these cases there is a possibility of deciding the question of disputed paternity.

As regards the nature of the agglutinable elements, it was also pertinent to demonstrate their antigenic nature. Accordingly the attempt was made to show that the specificity of the immune sera used in the tests is influenced not merely by the individuality of the animals producing the immune sera but likewise by the peculiarity of the injected blood. The experiments bear out this view although the antigenic capacity of two of the specific structures is rather low. In this they resemble the agglutinable property which was described by Schiff as occurring by preference in blood cells of group O. Possibly

<sup>3</sup> See Todd and White (1), and v. Dungern and Hirschfeld (6).

this weak antigenic activity is in some way related to the fact that normal human sera do not contain isoagglutinins for the new agglutinable structures, similar to those for A and B.

Is the serological differentiation of individuals a general law as the data collected may suggest, or an attribute of all or certain higher organized animals only? Are the marked groupings found by isoagglutination in man and some domesticated animals perhaps vestiges of a polyphyletic origin? Studies on a number of species will be necessary to settle these questions, and may throw light on the puzzling phenomenon of the four human blood groups. In this respect special interest is attached to the behaviour of the blood of apes. It was found that in contrast to the other animals examined, including lower monkeys, agglutinogens apparently identical with the human isoagglutinogens A and B occur in the blood of anthropoid apes and that, in each individual, ape blood could be assigned to one of the four groups; a new demonstration of the close biochemical relationship between anthropoids and man (13), and at the same time an intimation of the early origin of the group agglutinins. It follows that with regard to a certain serological characteristic the cells of an individual may be identical with those of another species but different from the cells of individuals of the same kind.

Only two of the groups were detected in chimpanzees; of 31 animals 5 belonged to group O, 26 to group A. The number of specimens examined is still too small to establish beyond doubt that the absence of B is a constant species property in chimpanzees, but certainly the high frequency of A is rather striking. In lower monkeys the definite taxonomic significance of an agglutinable property could be demonstrated. An agglutinin related to, though definitely distinguishable from the human B was found in twelve species of New World monkeys (Platyrrhinae) and in six species of lemurs, but was absent in the eighteen species examined of Old World monkeys (Cercopithecidae) (14).

Structures similar to two of the new agglutinable factors of human blood were shown to be present in the blood of chimpanzees; in several gibbons tests for one of these factors were negative (13). It may be, therefore, that the structures have been acquired in phylogeny later than A and B.

Passing to racial properties we have to deal with the important work of L. and H. Hirschfeld (15). They discovered the fact, and it was amply confirmed by others, that the numerical incidence of the blood groups is markedly different in various human populations although distant races may show somewhat similar percentage figures. In animal races analogous conditions seem to obtain. The most conspicuous examples are those of the American Indians with a great predominance of group O (91.3 per cent O, 7.7 per cent A, 1.0 per cent B) the Australian aboriginals with 46 per cent of group A and 54 per cent of group O, and Hindoos with 31.8 per cent O, 18.5 per cent A, 40.9 per cent B, and 8.7 per cent AB. Of course, findings of this sort do not enable one to establish the race of a single individual.

The differentiation of closely allied species could be made in several instances by proper agglutination tests. An illustration was afforded by studies on human and anthropoid blood (13). It is known that the proteins of these species can hardly be distinguished with the common precipitin reactions (16). When, however, rabbit immune sera for human erythrocytes are exhausted with the blood of chimpanzees, agglutinins remain, active for all samples of human red cells but not for chimpanzee blood. So far attempts have failed to make the diagnosis of races in a similar way, as for instance in the case of white or colored men. Consequently, if constant serological race differences exist at all they are apparently of a lower order than those that distinguish species.

Some additional information on the serology of human races was yielded by tests for the new agglutinogens mentioned, made with the blood of whites, American Indians and negroes. A statistical result was obtained similar to that of Hirschfeld's findings, with one of the agglutinogens. For this 1,636 white and 694 colored individuals (negroes) were examined. Of the former 19.2 per cent and of the latter 29.1 per cent lacked the property, a difference similar to that found for the isoagglutinin B in the same population. With another of the structures the disparity was still more pronounced. Two hundred and seven white and 231 colored individuals were examined. In these tests the reactions varied in intensity. The strongest agglutinations occurred, in 10.7 per cent of white and 33.5 per cent of colored men, weak reactions in 44.4 and 12.4 per cent respectively. These

observations seem to offer some prospect of a further serological characterization of races. The results will perhaps improve when purer blooded stocks are subjected to the test. Also the blood of American Indians was found to differ from that of white men with regard to the frequency of two of the agglutinogens.

If one tries to sum up the still scanty data it would seem that the serological diversity of the blood of various races is in general not brought about by the constant presence or absence of one or several qualities. One has rather to suppose that, in analogy to certain morphological features, there exists an average composition of the mosaic of characters around which the individual patterns fluctuate. Apparently a similar view holds for the serological species distinction of cells by agglutination except that there is as a rule a definite constant difference and less overlapping by individual variations.<sup>4</sup>

I propose to turn next to the nature of biochemical specificity. Since this question first arose, upon the evidence of serological research, it was by common consent assumed to be due merely to the diversity of proteins. Some doubt has been cast on this belief, and the existence of specific lipoids has been suggested by several observations such as the antilytic and immunizing capacity of alcohol or etherial blood extracts.<sup>5</sup> However, the subsequent experiments on the supposed lipoidal nature of some antigens were inconsistent and failed to carry the full conviction desirable in view of the importance of the subject. The aspect of the question was changed and certain discrepancies were settled as a result of studies on the heterogenetic antigen of Forssman. This substance can be separated by simple extraction with alcohol into a protein part, apparently responsible for the antigenic effect and another part, soluble in alcohol in its crude stage, that reacts specifically with immune serum without being antigenic by itself. As proof of this view it was found that the alcohol soluble substance can be converted again into an antigen. This succeeded unexpectedly by simply mixing it with protein solutions, the effect probably being caused by the formation of a loose compound with the protein.

<sup>4</sup> Cf. the results of Schiff and Adelsberger (17) on the relation between Forssman's antigen and the agglutinin A.

<sup>5</sup> Cf. the experiments of Landsteiner and v. Eisler (18), Much, Bang and Forssman (19), K. Meyer (20).

Further work showed that specifically reacting non-antigenic substances—so-called haptens—play a great part in the constitution of the antigens of animal cells and bacteria. For the latter this was demonstrated by Zinsser and Parker. Concerning the bacterial antigens a remarkable discovery was made by Avery and Heidelberger. They succeeded in isolating and purifying highly active substances reacting specifically with antibodies but devoid of antigenic properties, from pneumococci and capsulated bacilli, which turned out to be complex carbohydrates of acid character.

The chemical investigation of Forssman's hapten offered considerable difficulties owing to the small quantities of active substance present in the alcoholic organ extracts along with large amounts of various lipoids from which it cannot easily be separated, and, consequently, a great bulk of crude material had to be worked up. From the extract active fractions were isolated with peculiar properties. These products are soluble in water or weak alkali, practically insoluble in the common organic solvents and have a distinctly lower carbon content than the known lipoids. On hydrolysis they yield reducing sugar and components of lipoids. One may make the assumption that the specific substance belongs to a particular sort of lipoids (Landsteiner and P. A. Levene).

A study of the antigens of red blood cells indicated that they are analogous in nature to Forssman's antigens (21). The alcoholic extracts of red cells react specifically with anti-erythrocyte sera in flocculation and complement fixation tests and when they are injected into rabbits along with protein the formation of antibodies can readily be demonstrated. Similar experiments were carried out with organ extracts (Sachs and co-workers (24)). In this case immunization effects could be observed also with the organs of the same species as the animal injected, an observation used by Sachs for advancing a theory of the Wassermann reaction.

From the results quoted it appears that animals possess two sorts of specific substances, on the one hand simple proteins, on the other complex antigens consisting of a combination of haptens with proteins.<sup>6</sup> Making the necessary reservations the haptens, essential for the species

<sup>6</sup> A similar statement holds for bacillary antigens.

specificity of animal cells, may be regarded as lipoids. These views (Landsteiner and van der Scheer (21)) were confirmed by Bordet and Renaux (22).

Aside from the chemical study of antigens it was noticed that the specificity of the serological reaction of blood cells obeys other rules than does the species specificity of proteins as shown by precipitin reactions.

This led to the conclusion that there exists a two-fold system of species specificity in animals (23). In the case of the proteins the serological and chemical properties in general parallel closely the zoological relationship and the changes are gradual (Nuttall (16), Reichert and Brown (24)). Thus one could roughly construct the zoological tree merely on the basis of precipitin reactions.\* The specificity of cells is characterized on the contrary by the occurrence of so-called heterogenetic reactions which disclose the existence of similar structures in unrelated species and at a glance would indicate a mosaic structure. This is illustrated by the wide distribution of Forssman's antigen (e.g., in the blood of sheep, chickens, turtles (25, 26)), the group reaction of sheep erythrocytes and human blood of group A (Schiff and Adelsberger (17)) and in a general manner by the old observation, that normal hemagglutinins recovered from their combination with blood are regularly active for the cells of numerous even distant animals (27).<sup>7</sup> In this connection it is noteworthy that the occurrence or failure of agglutination when normal serum of an animal is tested with erythrocytes of another species is almost independent of the serological relationship.

A second distinguishing feature is the fact that allied species as a rule can be differentiated by hemagglutinins but not by precipitins. This is hardly caused by the unequal sensitivity of the two methods only, because with erythrocytes the said differences are considerable in comparison with those exhibited by distant species. It is true that a distinction of closely allied animals succeeds with precipitins too, by means of Uhlenhuth's (28) method of cross immunization, i.e., when the antibodies are derived from the species to be compared. On the

\* This remark refers to the studies on Vertebrates only.

<sup>7</sup> An example is the following: Agglutinin solutions obtained by warming rabbit cells agglutinated by beef serum, acted intensely on rabbit and frog erythrocytes.

other hand with the use of agglutinins, such results can be obtained (and even the discrimination of individuals be made) with common immune sera and strangely enough also with normal sera (29, 30, 23, 4). In general the degree of specificity of immune hemagglutinins is not greatly influenced by the species furnishing the antibodies while the opposite holds for precipitins (Uhlenhuth). Probably the high specificity of rabbit precipitins for the proteins of rodents and their low electivity toward bird proteins fall under this head.

Individual differences of cell antigens are easily demonstrable. On the other hand, the reports on individual or racial serological differences of serum proteins (31, 32) such as are believed to exist in analogy to the protein differences of species are not very well substantiated. Hence the question arises whether the opinion that proteins vary individually is correct or whether a definite chemical constitution of the proteins, e.g., of hemoglobin, is a constant species character. It has been assumed, in order to explain the group reactions of precipitins, that the proteins are mixtures, the elements of which may be present in several species (Arrhenius (33)). But this view is untenable on account of the studies on hemoglobin crystals (24, 34) which failed to demonstrate in a single sort of hemoglobin the multiplicity required by the hypothesis.

There can be no doubt as to the individual variations of normal antibodies, which are considered to be proteins or intimately connected with such. That the occurrence of these substances may be constitutionally determined became obvious by the very existence of human isoagglutinins and can be concluded from other instances especially the behavior of hemagglutinins and hemolysins. An example is the following. In examining the serum of monkeys it was found that the serum of *Macacus rhesus* agglutinates by preference human blood of group A, the serum of Vervet monkeys acts most intensely on blood of group B, while baboon serum reacted in one or the other way depending on the particular animal. However, the variations of these antibodies seemingly do not involve the structures which underlie species specificity and are limited to serum proteins and especially to certain fractions thereof.

That individual protein differences occur has been assumed also on the basis of purely chemical investigations. Meyer (35) and Treud-

tel (36) found differences in the flocculability and the isoelectric point of casein prepared from the milk of various women. The existence of more than one form of hemoglobin in the blood of one species was reported by Küster (37) and by Anson, Barcroft, Mirsky and Ouinuma (38). According to the claims of Falkenhausen and Fuchs (39) racial differences in proteins can be detected by the action of digestive serum ferments. It will be of importance to repeat and extend these studies and to ascertain whether the findings bespeak constitutional and hereditary variations of proteins among individuals, analogous to those which have been found to exist in the complex cell antigens or their haptens. If the answer is in the affirmative one should rather expect to obtain parallel results by serological reactions.

In closing it may be mentioned that the question just pointed out is of biological significance. Since species are characterized not only by morphological attributes but by their specific biochemical constitution as well, it seems evident that the somatic and functional development of the organic world was paralleled by a biochemical evolution of the proteins and haptens. One must assume that the two lines of events are linked in some way although no explanation has been offered as to how such a correlation might be brought about. In this regard it is essential to know whether the small initial steps in evolution are coupled with changes in the proteins. If so, individual and racial protein differences, perhaps too small to be detected by the methods available, ought to be a matter of regular occurrence. In the opposite case it is conceivable that the transformation of proteins came about discontinuously, contingent upon the occurrence of numerous changes or modifications of a special kind in the germinal constitution, perhaps in connection with hapten variations. It is too hazardous, attractive as it would be, to speculate further along these lines. Yet to perceive the problem may not be superfluous and may lead to experimental investigation.

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## BIOLOGICAL REACTIONS TO THE CHEMICAL FRACTIONS FROM HUMAN TUBERCLE BACILLI.\*

### II. THE IDENTIFICATION OF A SPECIFIC MATURATION FACTOR FOR MONOCYTES AND EPITHELIOID CELLS, AND AN ANALYSIS OF THE RÔLE OF THE MONOCYTE IN THE RESISTANCE TO TUBERCULOSIS.

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The reaction of the body to an infection with *living tubercle bacilli* represents a complex response of the cells of the blood and connective tissues. In addition to the pathognomonic lesion, the tubercle, there is an early involvement of the neutrophilic and eosinophilic leucocytes, with clasmatocytes, and an ultimate increase of lymphocytes and fibroblasts. The relative proportions of these cellular types in the general connective tissues is an index of the severity and progress of the disease.

The reaction of the body to the injection of *dead tubercle bacilli* shows the same mixed cellular types, and differs only in that the initial establishment of the specific tubercle never leads to the progressive disease found with the living organisms, and that the connective tissues pass through the cycles of a healing lesion.

Last year we reported<sup>1</sup> the results of some preliminary experiments with chemical fractions obtained from dead tubercle bacilli, carried out in co-operation with the Research Committee of the National Tuberculosis Association, of which Dr. William Charles White is Chairman. From Drs. T. B. Johnson<sup>2</sup> and R. J. Anderson,<sup>3</sup> of the Sterling Chemical Laboratory of Organic Chemistry, Yale University, we obtained the ether-insoluble and alcohol-ether-soluble fractions respectively, from strain H-37 of the human tubercle bacilli grown on Long's synthetic medium.

\* Work done in co-operation with the Committee on Medical Research of the National Tuberculosis Association.

The *proteins* 304 and 903, from Drs. Johnson and Coghill, produced definite toxic symptoms in rabbits; anorexia, loss of weight, temperatures reaching 108°F., extensive diffuse hemorrhage in organs and connective tissues, marked proliferation of clasmotocytes in the interstices of the lung septa, making the so-called interstitial pneumonia, and finally death. These results were confirmed this year with proteins 304-A and 304-B; they produced the same changes to a less marked degree, except that the rise in temperature was approximately as great. In no instance was there found the specific tubercle nor any increase in the monocytic strain of cells from which the epithelioid and Langhans giant cells have been shown to arise.

The alcohol-ether-soluble fractions, *phosphatides A-3* and *A-4*, from Dr. Anderson, produced none of the toxic symptoms and signs found with the protein. The rabbits remained in a thriving condition, but on post mortem examination there were found all of the cellular reactions in the connective tissues of the peritoneal cavity typical of the disease itself. Especially prominent were the monocytes and their derivatives, the epithelioid and Langhans giant cells making the pathognomonic lesion of the disease. This year we have followed rabbits over a period of five months after the administration of the phosphatide, and the typical tubercular tissue had been gradually absorbed precisely as in the case of the reaction to dead tubercle bacilli, with no embarrassment at any time to the animals. It is important to record that certain lots of the phosphatide A-3 kept for more than a year in the laboratory without special precautions have shown minor changes in the biological reactions correlated with changes in chemical properties.

In these studies it has been found that the peritoneal cavity, and particularly the peritoneal fluid and omentum, afford the most admirable material for studying the reaction of the tissues and cells to the various chemical substances, the former for differential counts of the cells called forth into the peritoneum and the latter for the study of the relationships of the cells stimulated in the tissues. The freshly exposed omentum may be spread as a thin film on a slide prepared with neutral red and Janus green, covered with a thin coverslip, and then studied, using the fine adjustment of the microscope to dissect the various levels of cells.

Since our last report, we have had from Dr. Anderson further fractionations<sup>8</sup> of the complex phosphatide A-3, as well as some of the remaining residues of the ether-soluble fraction from the bacilli. The tests of these substances here reported represent for the most part the reaction in the peritoneal cavity to one, two, and three doses at 24-hour intervals; in a few instances the more chronic reactions were studied. After trying various vehicles, such as olive oil and oleic acid, for these non-aqueous-soluble substances, a purified paraffin oil (Nujol) was found to be the least irritant per se to the peritoneum. It is of significance that no fraction from the tubercle bacillus thus far tested has been without some effect on the cells of the connective tissues.

The phosphatide A-3 yielded on hydrolysis (Dr. Anderson) three ether-soluble fractions, palmitic and oleic acids, and a liquid, saturated, fatty acid, together with certain water-soluble fractions. *Palmitic acid*, when injected into the peritoneal cavity, proved to be only a slight irritant to the cells of the omentum; there was an increase in neutrophilic leucocytes and young monocytes, and an accentuation of phagocytic activity of the clasmatoocytes. The *oleic acid* produced marked evidence in the gross of irritation of the tissues, the neutrophilic leucocytes and highly phagocytic clasmatoocytes predominating upon microscopic examination. Some increase of young and mature monocytes, but no epithelioid or giant cells were seen.

The liquid, saturated, *fatty acid (I)* from the phosphatide A-3 has been tested in 11 animals. In every instance but one the predominant reaction has been of the monocytic strain of cells toward the epithelioid and Langhans giant cell types (in one instance the reaction was of clasmatoocytes and monocytes in about equal proportions). After the first acute reaction of neutrophilic leucocytes and clasmatoocytes, which is minimal in the case of this fatty acid, the reaction tends more and more toward a pure strain of the cells typical of the disease, tuberculosis. In summary, the active principle for the proliferation and maturation of the epithelioid cells obtained with the phosphatide A-3 seems to be contained primarily and almost quantitatively within this saturated fatty acid obtained from it.

In addition to the phosphatide fraction, we have tested what have

been designated provisionally as *soft wax* and *purified wax* (white powder) from the alcohol-ether-soluble portion of the tubercle bacilli.

The *purified wax*, and an *unsaponifiable* fraction therefrom, both elicited a mixed response in the peritoneum of the normal rabbit; chemotaxis of neutrophilic leucocytes from the blood to the tissues and peritoneal fluid; irritation of the serosal cells; stimulation of the clasmatocytes in number and phagocytosis; and the formation of new monocytes with their maturation into typical epithelioid cells. From this purified wax, Dr. Anderson isolated a second liquid, saturated, *fatty acid (II)*. This second fatty acid showed only in a minimal degree the general irritant reactions of the purified wax. The predominant reaction was an unequivocal stimulation to the monocytes, with the development of very many typical epithelioid and Langhans giant cells in both omentum and peritoneal fluid.

The *soft wax* was predominantly irritant, the neutrophilic leucocytes filling omentum and peritoneal fluid, and the serosal cells and clasmatocytes showing marked stimulation. In certain preparations, many mitotic figures were seen in the clasmatocytic cells. However, there was also a definite, though minimal, stimulation of young monocytes and the appearance of a few epithelioid cells. From the soft wax another *fatty acid (III)* was isolated, which again evidenced a minimal irritant reaction with the preservation quantitatively of the maturation factor for monocytes and epithelioid cells.

In summary, 15 rabbits, receiving intraperitoneal injections of a liquid, saturated, fatty acid (I, II, or III), isolated from the ether-soluble portion of the tubercle bacillus, have all, without exception, shown a characteristic cellular response in a relatively pure state. That is to say, these fatty acids have caused the production of new young monocytes, with the markedly basophilic cytoplasm characteristic of the beginning maturation of the immature white blood cells, and have further stimulated their development into mature monocytes with subsequently the metamorphosis into typical epithelioid and Langhans giant cells. This is the first maturation factor identified for any of the white cells.

The aqueous-soluble *polysaccharide*, obtained by Dr. Anderson from the moist bacteria in connection with the extraction of the lipoids with alcohol and ether, has been tested. This polysaccharide, when

given intravenously in normal rabbits over a period of days, produced no characteristic change in the blood picture, and on post mortem examination there were no lesions in the tissues or organs. When given intraperitoneally in rabbits, the predominant reaction was of the neutrophilic leucocytes, with a marked increase in the number and activity of the clasmatoocytes. No evidence of stimulation of monocytes toward the epithelioid type was seen. In tuberculous guinea pigs, as first pointed out by White, 10 mgm. of the polysaccharide given intraperitoneally may cause death within a few hours. A marked leucopenia of all of the cells of the blood is induced, the lymphopenia persisting for twenty-four hours, and the neutrophiles producing a leucocytosis within four to six hours. Depending upon the extent and severity of the tuberculosis, there is either a transient rise in temperature of 1° to 3°F., with eventual recovery, or a steady fall in the temperature is noted to as low as 94°F., with collapse and death. The same phenomena are observed with the protein 304, and further studies are now in progress in an attempt to analyze the meaning of this reaction.

The rôle of the lymphocyte as a factor in resistance to tuberculosis has been emphasized by many investigators. We have been able to show that an increase in the normal ratio of lymphocytes to monocytes in the peripheral blood is correlated with receding lesions. It is therefore significant that no substance so far isolated from the bacillus itself has caused any increase in lymphocytes in the tissues, but that, on the contrary, the polysaccharide and protein have definitely depressed the lymphocytes of the blood in both normal and tuberculous guinea pigs.

Having established first in experimental tuberculosis<sup>4</sup> that the monocyte-lymphocyte ratio in the blood may be taken as an index of the progress and extent of the disease, with ample clinical confirmation<sup>5,6,7</sup> now available, and with the report here of the isolation from the phosphatide partition of the tubercle bacillus of a specific maturation factor for the cells which make up the characteristic lesion of the disease, the tubercle, it becomes necessary to try to evaluate the rôle of this strain of cells in tuberculosis. With this end in view, we have carried out two series of experiments.

In one series of animals it was possible to decrease the number of

monocytes in tuberculous rabbits by the use of a specific anti-serum for rabbit monocytes developed in this laboratory. In a group of 22 rabbits so treated, 14, or 63 per cent, survived an intravenous dosage of 1 mgm. of bovine tubercle bacilli, strain B-1, for a period exceeding two months, and up to 13. In the control group of 42 rabbits receiving an identical dosage of the same strain of organisms, 5 only, or 11 per cent, survived beyond two months.

Conversely, a group of 7 rabbits have been given a series of injections, in dosages of 20-80 mgm. of the phosphatide A-3 to increase greatly the monocyte-epithelioid cells. This was followed by the injection of 1 mgm. of the organisms cited above. Six of these animals, or 85 per cent, are now dead, as contrasted with only 2, or 28 per cent, of the 7 control rabbits, and the experiments have been going from six to eight weeks at the present writing. Five of the 6 phosphatide animals died before either of the two controls, the earliest death being at 14 days following the infection, others dying at 17, 20, 21, 23, and 44 days, with lesions of much greater extent than those found in the two controls dying at 26 and 31 days. Two rabbits receiving less of the phosphatide (each dose 10 mgm.) and showing only a minimal response in the tissues, have survived 43 days to the present time, together with their two controls. The extent of the specific reaction to the phosphatide is directly proportional to the dosage.

These two series of experiments indicate that the monocytic strain of cells cannot be considered as a protective factor in tuberculosis.

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## CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

### IV. INFLUENCE OF LIGHT ENVIRONMENT ON NORMAL RABBITS.

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By a systematic study of the blood of normal rabbits (1), it was found that both the absolute and relative amounts of calcium and inorganic phosphorus exhibited periodic variations and that, among animals living in the laboratory, there was evidence of a tendency toward a progressive increase in calcium and of a decided decrease in inorganic phosphorus of uncertain duration and extent. It appeared that, while a number of factors may have contributed to the production of the variations noted, one of the chief causes was the occurrence of changes in the light environment.

It is, of course, well known that ultra-violet radiations of short wave-length (3100 Ångström units or less) are capable of exerting a profound influence on the inorganic phosphorus content of the blood and hence on the calcium-phosphorus equilibrium. In the present instance, however, it was evident that the variations found among animals living in the laboratory could not be attributed to the action of ultra-violet light of short wave-length as the light received by these animals was filtered through glass.

From previous experiments (2), it had been found that rabbits living under similar conditions showed periodic variations in the weights of organs, or organic constitution, and in their reaction or susceptibility to disease, and that these conditions were subject to the influence of a light environment which included no ultra-violet of short wave-length. It seemed probable, therefore, that blood calcium and inorganic phosphorus might be influenced by similar

conditions. This assumption was supported by the fact that Mayer-son, Gunther, and Laurens (3) found that, in dogs, calcium and phosphorus were affected by a change from room light to darkness or *vice versa*, and they emphasized the fact that the results obtained by them were similar in character to effects produced by irradiation with the carbon arc.

In studying the influence of light environment on the calcium and inorganic phosphorus content of the blood, our chief interest was in effects of conditions involving light of long wave-length and a minimum of radiant energy as compared with the intensity of illumination, that is, in conditions as far removed from the ultra-violet end of the spectrum as possible. The experiments carried out were based on a comparison of certain reactive phenomena presented by animals living under the usual laboratory conditions, in total darkness, or in an environment of neon light. In addition to calcium and inorganic phosphorus, parallel observations were made on the effects of the conditions mentioned on nutrition and growth, the growth of hair, on organic constitution, and on the reaction to disease. The results for the first three phases of the investigation have been reported (4); those for calcium and inorganic phosphorus will be presented in the present paper.

### *Material and Methods.*

The animals used for the experiments to be reported were the same as those used for the study of the influence of light on nutrition and growth, the growth of hair, and organic constitution. The conditions of the experiments were given in detail in the first of these papers (4) but may be repeated in part for the sake of clarity.

The experiments to be reported represent parallel observations on rabbits living under conditions which differed only with respect to the light environment. One group of animals, which for convenience may be designated as the controls, was kept in a well lighted (sunlight), well ventilated room with a southern exposure. The room was heated during cold weather and an effort was made to maintain a temperature of 60–70°F. (thermostatic regulation). These animals are designated as controls merely because they represented the usual as contrasted with an unusual condition.

A second group of rabbits was kept in a room from which all light was excluded. Other living conditions were the same as those of the first group. During the course of the experiments, these animals received some light from two sources. For a brief period each day, a 30 watt Mazda lamp was used in this room for clean-

ing cages, feeding, and making the necessary observations. From time to time, the animals were also brought into the laboratory for bleeding and were thus exposed to diffuse filtered sunlight for a period which rarely exceeded an hour per week.

A third group of animals, the light group, was kept in a room similar to the others, with sunlight excluded. This room was lighted continuously by 20 standard neon tubular lights (crown glass), 6 feet in length and 5/8 inch in diameter. The tubes were mounted in an upright position on a rectangular frame in the center of the room, parallel with and facing the cages at a distance of approximately 3½ feet. The lights were operated on a current of 25 milliamperes at 8000 volts to a bank of 3 lamps in series.

The terms control, light, and dark are used with reference to the three groups of animals in a purely descriptive sense. Each group was intended to supply information concerning the biological action of a particular kind of light environment and each served as a control to the others.

As is well known, the light produced by neon Geissler tubes varies from a rose-red to a bluish pink color, depending upon the condition and the operation of the tube. The heat produced is very slight. The rays transmitted by crown glass are concentrated in two regions with faint bands intervening. The shortest rays lie between 3370 and 3620 Ångström units. The strongest of these are in the region of 3460 and 3480. The greater part of the light produced is of very long wave-length and is composed of rays from about 5800 to the lower limit of registration at 7600 Ångströms. The vertical illumination at the front of the cages varied between 21 or 23 foot candles at the ends of the rack and 54 at the center with a mean of 34.3 foot candles. No attempt was made to measure the energy delivered by this equipment as it was perfectly obvious that such measurements would serve no particular purpose in the present instance.

We wish also to emphasize the fact that the animals used in these experiments were given no special preparation to facilitate the action of light such as clipping or shaving of hair.

Observations were made on 3 groups of normal male rabbits living under the conditions described. At the beginning of the experiments, the animals were 8 to 10 months old; they were sexually mature but had not attained their full growth. In each case, the animals for a given experiment were assembled and divided into 3 comparable subgroups according to type or breed, age, and weight, but without any knowledge of the calcium or phosphorus content of the blood. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage.

Group I contained 15 albino rabbits, Group II, 15 black rabbits, and Group III, 15 gray, brown, or black rabbits. In each case there were 5 animals exposed to the neon light, 5 in the dark, and 5 received diffuse, filtered sunlight of varying intensity and duration.

The animals of Groups I and II were placed under observation Oct. 1, 1926,

and kept under control conditions until Oct. 22. Determinations of calcium and inorganic phosphorus were begun Oct. 8 and continued at weekly or biweekly intervals until May 18, 1927. 323 calcium and 321 phosphorus determinations were made on Group I and 329 and 327 on Group II.

The animals of Group III were also placed under observation Oct. 1, 1926, and were put into their experimental quarters at that time, but no blood analyses were made until Dec. 1. The blood of these animals was examined at irregular intervals and on Feb. 8, 1927, the light and dark divisions of the group were interchanged so that animals that had been living in an environment of neon light were placed in the dark and *vice versa*. This condition was maintained until May 4, when the two groups were restored to their original environmental conditions and observations were continued until June 8, 1927.

For this group of animals there are 3 sets of results: first, a series of 20 blood analyses on each of the 3 subdivisions, most of which were made during the last month of a 4 months period of continuous exposure to neon light or confinement in the dark with corresponding observations on the controls; second, a series of 45 blood analyses covering a period of nearly 3 months during which the light environment of the experimental divisions was reversed; and a series of 20 analyses covering a final period of approximately 1 month during which the animals were restored to their original environmental conditions. The total number of calcium and of phosphorus determinations made on the 3 groups of animals was 907 and 903 respectively.

The methods employed in making determinations of calcium and inorganic phosphorus were described in detail in the first paper of this series (5).

The results for calcium, inorganic phosphorus, and the ratio of calcium to inorganic phosphorus are recorded in Tables I to IV as group means for consecutive determinations. The values for calcium and phosphorus are given in mg. per 100 cc. of serum.

The general trend of the variations observed and the relative magnitude of the values obtained are shown graphically in Figs. 1 to 18. These curves were obtained by smoothing the group means as given in Tables I to IV by the formula  $\frac{A + 2B + C}{4}$  and converting the smoothed values into percentage deviations from the mean values obtained for a large series of determinations on normal rabbits (5).

## RESULTS.

The results of the experiments are summarized in Tables I to IV and Figs. 1 to 18.

TABLE I.  
*Calcium. Group Means for Consecutive Determinations.*

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
	mg	mg	mg	mg.	mg	mg
Oct 8	13 66	13 8		13 8	13 7	14 2
" 15	13 15	15 1	14 3	13 3	14 0	13 8
" 21	15 79	14 0	15 0	15 0	13 6	16 0
" 29	15 43	13 9	15 3	14 9	13 1	15 0
Nov. 5	16 50	16 5	15 2	15 6	16 2	16 5
" 12	13 68	14 2	14 1	14 2	13 9	15 0
Nov 19	15 16	15 0	15 3	15 2	14 6	15 9
" 26	14 62	14 7	14 8	14 7	14 0	14 9
Dec 3	15 35	15 3	15 7	15 2	14 8	15 9
" 10	15 51	15 5	15 7	15 5	15 6	15 5
" 17	16 12	15 5	15 7	14 8	15 5	15 8
" 31	15 77	16 0	16 5	16 1	15 4	15 5
Jan. 14	15 40	15 2	16 3	15 4	15 5	17 0
" 28	16 68	16 2	15 8	17 1	16 3	16 2
Feb. 11	15 20	15 2	16 1	15 3	15 0	16 1
" 25	15 56	15 8	16 1	15 6	14 6	16 2
Mar. 11	16 04	15 8	16 1	15 5	15 3	15 8
Mar. 25	15 84	15 4	15 0	15 4	15 2	15 9
Apr. 8	15 23	15 5	15 3	15 3	14 8	16 1
" 22	16 51	15 6	15 1	15 1	14 5	15 6
May 4	15 43	15 0	14 5	15 5	15 1	14 6
" 18	17 18	17 5	17 0	18 3	16 4	17 1

TABLE II.  
*Inorganic Phosphorus. Group Means for Consecutive Determinations.*

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
	mg.	mg.	mg.	mg.	mg.	mg.
Oct. 8	4.41	5.48		5.95	5.80	4.09
" 15	4.47	3.79	4.17	4.63	3.48	4.73
" 21	3.39	4.22	3.98	4.91	4.55	5.19
" 29	4.00	4.43	4.24	4.31	4.50	4.76
Nov. 5	4.67	4.32	4.22	4.87	5.05	4.79
" 12	4.73	5.29	4.63	5.31	5.39	4.73
" 19	4.90	4.27	4.63	5.06	4.78	4.49
" 26	4.39	4.71	4.56	5.01	4.86	4.65
Dec. 3	4.61	4.70	3.99	4.43	4.19	3.98
Dec. 10	4.18	4.24	4.38	4.33	4.48	4.21
" 17	3.61	4.23	4.38	4.59	3.91	4.09
" 31	4.40	3.56	4.39	4.72	4.12	4.25
Jan. 14	4.19	4.48	4.02	4.50	4.55	3.91
" 28	3.90	3.55	3.66	3.98	3.61	3.90
Feb. 11	4.14	4.01	4.06	4.15	4.35	3.99
Feb. 25	4.21	4.05	3.76	4.08	3.98	3.97
Mar. 11	3.86	3.41	3.39	3.92	3.50	3.52
" 25	3.81	3.67	3.65	3.88	3.87	3.70
Apr. 8	3.95	3.62	3.17	3.56	3.69	3.41
" 22	3.56	3.50	3.10	3.32	3.25	2.85
May 4	3.39	3.37	3.24	3.13	3.51	3.20
" 18	3.93	3.88	3.85	3.96	3.88	3.75



TABLE III.

*Calcium-Phosphorus Ratio. Group Means for Consecutive Determinations.*

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
Oct. 8	3.11	2.52		2.32	2.36	3.47
" 15	2.95	3.98	3.43	2.87	4.02	2.92
" 21	4.66	3.32	3.77	3.06	2.99	3.08
" 29	3.85	3.14	3.61	3.46	2.91	3.15
Nov. 5	3.73	3.82	3.60	3.20	3.21	3.45
" 12	2.90	2.68	3.05	2.67	2.58	3.17
" 19	3.10	3.51	3.31	3.00	3.05	3.54
" 26	3.33	3.12	3.25	2.93	2.88	3.20
Dec. 3	3.34	3.26	3.94	3.39	3.53	4.00
" 10	3.71	3.66	3.59	3.58	3.48	3.68
" 17	4.46	3.66	3.59	3.22	3.96	3.86
" 31	3.59	4.49	3.76	3.41	3.74	3.65
Jan. 14	3.68	3.39	4.06	3.42	3.46	4.35
" 28	4.28	4.56	4.32	4.30	4.52	4.15
Feb. 11	3.67	3.79	3.97	3.69	3.45	4.04
" 25	3.71	3.93	4.28	3.82	3.67	4.08
Mar. 11	4.15	4.63	4.75	3.95	4.37	4.49
" 25	4.15	4.20	4.11	3.97	3.93	4.30
Apr. 8	3.85	4.28	4.83	4.30	4.01	4.72
" 22	4.64	4.46	4.87	4.55	4.46	5.47
May 4	4.54	4.45	4.48	4.95	4.30	4.56
" 18	4.38	4.51	4.42	4.62	4.23	4.56

TABLE IV.  
*Group III. Group Means for Consecutive Determinations.*

Date	Calcium			Phosphorus			Calcium-phosphorus ratio		
	Control	Light	Dark	Control	Light	Dark	Control	Light	Dark
	mg.	mg.	mg.	mg.	mg.	mg.			
Dec. 1	13.9	14.0	15.0	3.83	3.98	3.71	3.64	3.51	4.05
Jan. 5	15.3	14.7	16.6	5.03	4.81	3.77	3.04	3.05	4.41
" 19	15.6	15.9	16.5	4.66	4.33	4.04	3.34	3.67	4.08
Feb. 4	17.2	17.2	18.4	4.38	4.27	4.13	3.93	4.04	4.44
Feb. 11	16.6	15.3	15.2	4.41	3.85	4.46	3.72	3.97	3.42
" 16	15.4	15.4	14.9	4.32	4.07	4.10	3.55	3.77	3.63
" 28	15.6	15.5	15.2	3.85	3.66	4.10	4.04	4.25	3.70
Mar. 2	15.0	15.5	15.3	4.07	3.95	4.19	3.67	3.92	3.65
" 9	14.7	14.6	15.9	4.10	3.84	4.28	3.58	3.80	3.71
" 16	14.8	15.2	15.6	3.64	3.37	3.53	4.06	4.51	4.41
" 23	14.8	14.5	15.4	3.72	3.85	3.82	3.96	3.77	4.03
Apr. 6	15.5	15.0	15.5	4.53	3.81	4.11	3.41	3.94	3.77
May 4	15.4	14.4	15.9	3.87	3.28	3.53	3.98	4.40	4.51
May 6	15.2	15.1	15.3	4.21	3.59	4.17	3.62	4.20	3.66
" 11	17.0	16.1	16.3	3.90	3.50	3.83	4.35	4.60	4.27
" 25	16.6	16.6	16.9	3.76	3.71	3.72	4.41	4.47	4.54
June 8	15.8	16.7	16.0	3.61	3.37	3.56	4.36	4.95	4.48

## DISCUSSION AND CONCLUSIONS.

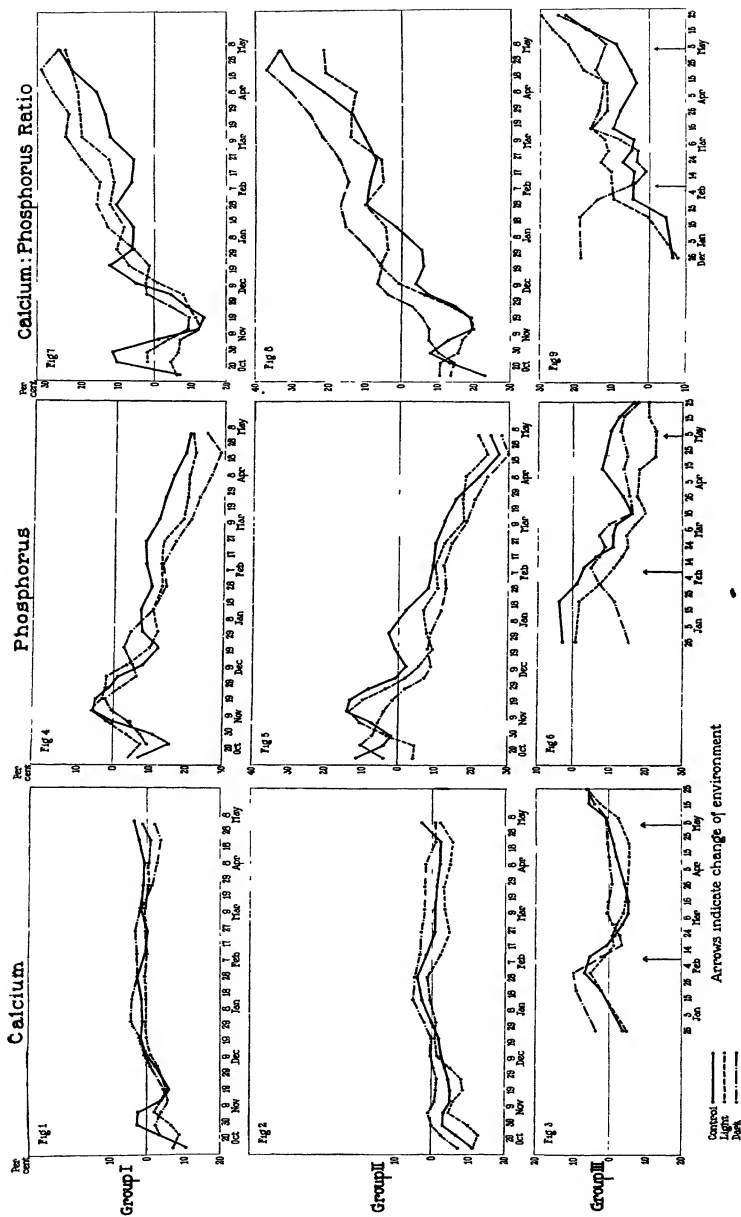
Analysis of the results will be facilitated if we consider certain features of the reaction shown by the smoothed curves in Figs. 1 to 18 before attempting to make a comparison of actual values.

An examination of the curves for calcium and inorganic phosphorus of Groups I and II (Figs. 1, 2, 4, 5) shows that the changes found in the blood of these animals are divisible into several phases. First, there was a period during which wide variations occurred from one examination to the next. This condition lasted from the beginning of the experiments until about Nov. 19 or 26, or for a period of 7 to 9 weeks. During the next month, the changes that occurred were more gradual and assumed a more orderly progression in a given direction. In general, calcium increased and inorganic phosphorus decreased. This was followed by a third period of 6 to 8 weeks during which there was comparatively little change of level or, in the case of calcium, there was a gradual reversal of the direction of the curves. Finally, at the end of 4 to 5 months, the magnitude of the variation increased again with a decrease in both calcium and phosphorus which changed to an increase as the experiments were concluded.

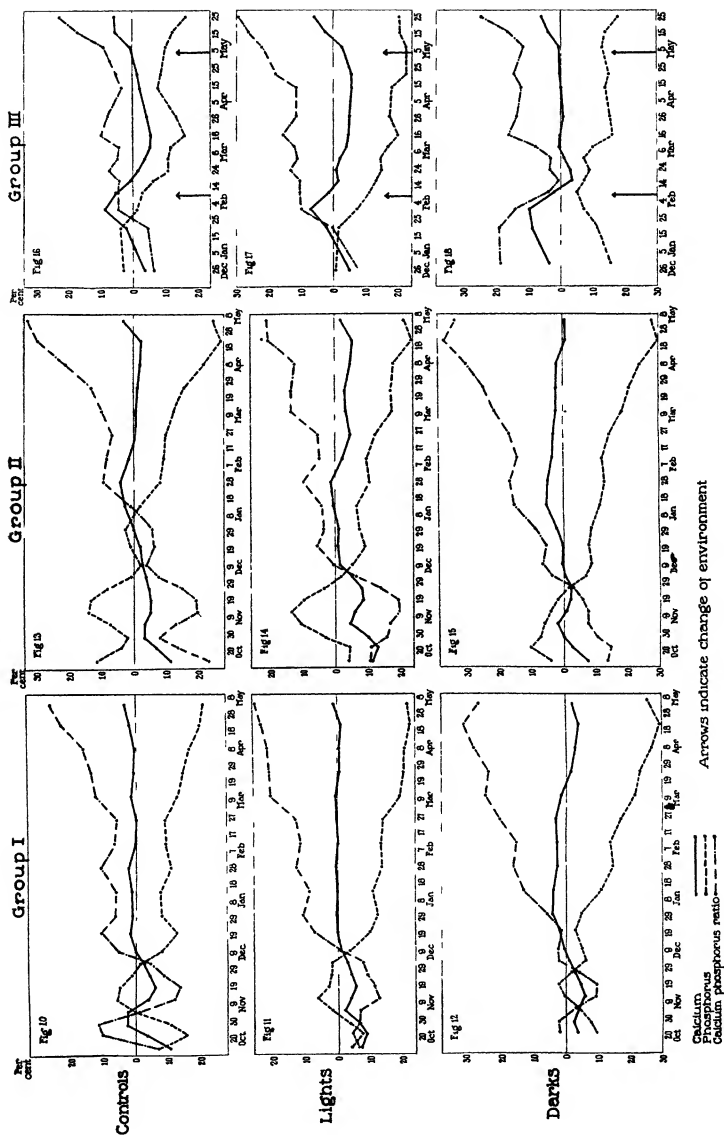
It will be noted that variations of this general type were shown by control, light, and dark animals. There were, however, distinct differences in the magnitude and direction of the variations that occurred as well as in the time relations, while the results for Group I were not identical with those for Group II.

The problem presented is to determine whether these variations in the calcium and phosphorus content of the blood were influenced to an appreciable extent by light environment and, if possible, the nature of the influence exerted by a particular environmental condition.

The results obtained for control animals during the first 7 to 9 weeks are clearly indicative of a profound disturbance of the calcium-phosphorus equilibrium. As was pointed out in a previous paper (1), a change of this kind is almost invariably produced by a change from outdoor to indoor life. Mayerson, Gunther, and Laurens (3) found that similar disturbances of the calcium-phosphorus equilibrium were produced in dogs by a change from room light to darkness or *vice versa*, or by irradiation with the carbon arc. It seems, therefore, that the first series of variations which occurred in the blood of control animals



FIGS. 1 TO 9.



Arrows indicate change of environment

Figs. 10 to 18.

may be regarded as a reaction to the change in the mode of living in which the light environment, and the deprivation of ultra-violet in particular, was an important factor.

Animals of the light and dark divisions were subject to the same influences as the controls for a period of 3 weeks, and they show evidence of the occurrence of changes similar to but not identical with those of corresponding controls. Among the white animals of Group I, the increase in calcium during this period was distinctly less. This is particularly true of the dark division which showed a decrease rather than an increase during the time that the values for control animals were at their maximum (Fig. 1).

The results for the black animals of Group II were different (Fig. 2). The increase in calcium for controls was less than that of Group I, but the results for the dark division virtually paralleled those for the controls at a slightly higher level, while the change in the light division was essentially the same as for Group I.

The results for inorganic phosphorus show a similar situation (Figs. 4 and 5). There was first, a decrease, and then a marked increase and a second decrease in which the animals of the light division followed the controls fairly closely. Among the albino rabbits in the dark, the reaction followed a similar course but the increase was less, as in the case of the calcium, while the black animals (Group II) showed a marked decrease in phosphorus during the period of increase in the control and light divisions.

One effect of these changes on the calcium-phosphorus equilibrium is shown in Figs. 7 and 8. In the case of Group I, it will be seen that the differences produced in the ratio of calcium to inorganic phosphorus during this first period of adjustment or accommodation to indoor life and to exposure to neon light or confinement in the dark are both qualitative and quantitative.

The effect on the controls was a marked increase in the ratio, followed by an even greater decrease, while the ratios for light and dark animals remained virtually constant during the period of increase, and the subsequent decrease was less than that shown by the controls. Results of a similar character were obtained for Group II, but in this case, after a slight drop in the calcium-phosphorus ratio, the animals in the dark showed a marked rise which was maintained during the

period of secondary decrease shown by control and light animals. This increase in the calcium-phosphorus ratio is, of course, influenced more by the reduction in inorganic phosphorus than by the increase in calcium which occurred at this time.

From a comparison of the results for this period, it seems that the most obvious effect of exposure to neon light or complete exclusion of light was to diminish the reaction of accommodation to indoor life as shown by control animals. While the actual influence exerted by the experimental conditions is more or less obscured by the reaction of accommodation, it seems that, in general, exclusion of light tended to produce a reduction in both calcium and inorganic phosphorus and that the influence of neon light was to depress the one and to increase the other. The effect of exclusion of light was greater than that of light of long wave-length. It also appears that the black animals were more susceptible to the influence of these conditions than the albinos; this is particularly true of the influence on inorganic phosphorus, but there is a suggestion that, in the case of calcium, the color relation may have been reversed.

During the second period of the experiment, the calcium in the blood of control animals increased; in Group I, it reached a maximum toward the end of December, but in Group II the rise continued until the end of January (Figs. 1 and 2). There was a similar increase in calcium among light and dark animals with the establishment of a definite order of magnitude, the animals in the dark giving the highest values, those in the light the lowest, and the controls occupying an intermediate position. The differences in level were comparatively slight, but were greater in the case of black than of white animals which is of interest in comparison with the results for Group III.

No blood analyses were made on the animals of Group III until Dec. 1, or until they had been under observation for 2 months. The results begin, therefore, with the period corresponding with the period of increasing calcium in Groups I and II, and we find precisely the same relations in Group III (Fig. 3) but with an even greater difference between the control and light animals on the one hand and dark animals on the other.

As calcium increased in Groups I and II, inorganic phosphorus decreased, and, in Group II, the decrease shown by animals of both

the light and dark divisions was greater than that of the controls. Among the control animals, the decrease in inorganic phosphorus was interrupted by a clearly defined secondary rise and a little later there was a slight increase among the animals of the light division, while in the dark division there was merely a reduction in the rate of decrease.

The relative order of magnitude of values for inorganic phosphorus was not exactly the reverse of that for calcium, but the lowest values were given by animals in the dark and the highest by the controls with the light division in the intermediate position.

The reaction of the white animals of Group I was somewhat different. In the first place, the high values reached during the preliminary period of adjustment were maintained for a longer time and the subsequent decrease in phosphorus was more gradual. This was particularly true of animals in the light division which maintained a higher level during this period than the controls. The reaction of animals in the dark was also peculiar in that they showed a secondary rise in inorganic phosphorus, the maximum of which coincided with the minimum of the controls, so that during the second half of the period, the relative position of these animals changed from lowest to highest.

The results for Group III (Fig. 6) agree with those for Group II in so far as the relative amounts of inorganic phosphorus in the blood of the 3 classes of animals are concerned and with even greater quantitative differences. But, in Group III, phosphorus rose in the blood of animals in the dark, while it decreased in the control and light divisions as in Group I.

Considering the 3 groups of animals, it is again evident that the general trend of the curves for calcium and inorganic phosphorus were essentially the same irrespective of differences in environmental conditions. There were, however, differences in the order of magnitude of the values obtained, and in the case of phosphorus, definite differences in the direction of the curves at certain times. In general, the evidence indicates that complete exclusion of light tended to diminish the inorganic phosphorus content of the blood and to increase the calcium especially in animals of a dark color, but was less effective in the case of albino rabbits. There is also evidence that neon light exerted a similar influence on the phosphorus content of the blood, but its action differed from that of complete darkness in that the



depression of the phosphorus was less while the calcium content of the blood was diminished and not increased.

The effect of these changes on the calcium-phosphorus equilibrium (Figs. 7, 8, and 9 and Tables III and IV) was to increase the ratio of calcium to inorganic phosphorus of all classes of animals in Groups I and II and of the control and light divisions of Group III. The dark division of the third group was first increased to a level far above that of control and light animals, and then diminished.

The relative magnitude of the change varied in different groups of animals. In Group I (albinos), the controls gave the highest value for this period, the light division came next, and the dark animals were lowest despite the fact that during the first half of the period the ratio was highest in these animals. In Groups II and III (dark colored animals) the order of magnitude was reversed with a ratio of calcium to phosphorus that was much higher in dark than in either the light or control animals, while the values for the light divisions were much higher than those for the controls.

Following the period of increasing calcium and decreasing phosphorus, the calcium level in control animals of Group I remained high for about 3 months. At the beginning of this period inorganic phosphorus increased slightly and then remained comparatively constant for about 2 months.

The values obtained for calcium in light and dark animals differed from those of the controls in that they showed less fluctuation and were at slightly lower and higher levels. There was a stabilization of inorganic phosphorus in the light animals during the latter part of the period, as in the controls, and a suggestion of a similar condition in the dark division, but on the whole, there was a continuation of the downward movement which was more marked in dark than in light animals.

The results for Group II differed somewhat from those for Group I. In the first place, the period of increasing calcium was more prolonged and this was followed by a definite decrease which was associated with a marked decrease in inorganic phosphorus. Light and dark animals reacted in a similar manner. There was, however, less reduction in the calcium of dark animals, while the light division showed a slight rise following an initial reduction of considerable duration and extent.

The relative magnitude of the three values was the same, however, as for Group I.

Following the increase of inorganic phosphorus in control animals of Group II (December) there was a marked decrease over a period of about 1 month. The decrease then continued at a rate virtually parallel with that of animals in the dark, but at a distinctly higher level. The animals in the light also showed a reduction in inorganic phosphorus, but in this case, the decrease was interrupted by brief periods of slight increase or stabilization.

During the last 2 months of the experiments, there were several changes in the relative magnitude of values. For some time, animals in the dark had shown higher values for calcium than either the controls or those in the light, but among the dark animals of Group I, calcium decreased until it was at a distinctly lower level than in either of the other divisions. In Group II the reduction occurred later, and when the experiments were concluded, the calcium level for dark animals was still slightly higher than that of animals in the light but lower than the controls.

Inorganic phosphorus decreased in control and dark animals of both groups with no material change of relation, but the decrease was more marked in Group II than in Group I. Among the animals in the light, the decrease was less; the difference between the light and dark divisions was increased, while in Group II the phosphorus level was higher than that of the controls with an evident tendency in the same direction in Group I.

As the experiments were concluded, all values were increasing again with the exception of the calcium in the dark division of Group II and the phosphorus of control animals of Group I.

The entire series of changes described above is presented in Figs. 10 to 15 from the point of view of the relation between the changes that occurred in calcium to those in inorganic phosphorus and their effect on the calcium-phosphorus equilibrium as indicated by the ratio between the two substances. We cannot attempt a detailed discussion of these curves, but it will be seen that while the relations presented assume the same general form in all cases, the exact relations are different as indicated by the spacial relations and directions of lines. The difference between the equilibrium shown by control and light animals is, on the whole, less than that shown by dark animals in comparison with

other groups. There are, in fact, 3 types of curves of which the one presented by animals in the dark is most distinctive. A comparison of results from this point of view shows that differences in absolute amounts of calcium or phosphorus, which might appear to be insignificant, may produce a very definite difference in the equilibrium between the two substances.

Another feature of the results to be considered is the effect of a change of environment on animals that have lived for several months in a fixed environment of neon light or in darkness as indicated by the results obtained for Group III (Figs. 3, 6, 9, and 16 to 18). When the first change was made (Feb. 8), calcium had reached a high level in all classes of animals, while inorganic phosphorus was decreasing in animals of the control and light divisions and, in the dark, increasing from an exceptionally low level.

Following the change of environment, there was a marked decrease in the calcium of the animals transferred from dark to light and a smaller decrease among those transferred from light to dark, but the controls also showed a decrease which continued for some time, while there was a slight and transient secondary rise among the animals that had been placed in the dark and a more decided and more prolonged rise among those that had been transferred from the dark to the light. This increase in calcium was maintained while the calcium of animals placed in the dark decreased to a level below the controls. Meantime, there was a gradual increase of calcium in control animals. After a brief period of adjustment there was thus a return to the relation that existed between light and dark animals at the time of the change in environmental conditions with the reestablishment of fairly stable levels, while the controls showed changes which were not reflected to an appreciable extent by either the light or dark divisions.

The immediate effect of the change of environment on the phosphorus is uncertain. There may have been an accentuation of the decrease in inorganic phosphorus of animals placed in the dark and a corresponding tendency toward a sustained elevation among those transferred to the light. At any rate, there was a change in the relative levels of the two groups of animals, and after a further decrease in inorganic phosphorus, the animals placed in the light showed an increase while the decrease continued among those in the dark.

When the second change of environment was made (May 4), both calcium and phosphorus were at lower levels than at the time of the first change. Calcium increased in both the light and dark animals, while inorganic phosphorus diminished in the dark group and increased slightly in the light, but it is not certain that these changes in the chemical composition of the blood can be regarded as an effect of the change in environment as the period of observation was too short to afford an opportunity for a definite determination of results.

If we compare the effect of this series of changes on the calcium-phosphorus equilibrium, as shown in Figs. 16 to 18, with conditions presented by the animals of Groups I and II (Figs. 10 to 15), it will be seen that, following the initial disturbance of equilibrium, the curves for both light and dark animals were more analogous to those of the light divisions of other groups than to the dark divisions. This is shown by the tendency to maintain a comparatively constant ratio. There is, however, a suggestion of the persistence of a dark effect in the high calcium-phosphorus ratio of dark animals while the tendency of this value to decrease rather than to increase is indicative of a light effect. It is to be noted that this decrease in ratio values occurred despite the persistence of a high calcium level, which may be regarded as a dark effect, and was due to an increase in inorganic phosphorus which is indicative of a light effect. The converse is true for animals that were transferred from a light to a dark environment so that, comparing the spacial relations of the three values shown in these figures with corresponding curves for Groups I and II, it appears that, despite the persistence of certain conditions which may be attributed to preexisting environmental influences, the change of environment reversed the general relations of the curves.

Thus far, the discussion has been confined to a consideration of the trend of the curves for calcium and inorganic phosphorus and such differences in results as are shown by comparisons of smoothed curves. It is evident that all classes of animals showed actual variations in calcium and inorganic phosphorus comparable with the periodic and progressive variations reported in a previous paper (1), so that the evidence bearing on this phase of the results need not be considered further. In addition, there is definite evidence of quantitative and qualitative differences between the results obtained for animals living under different environmental conditions as well as for animals

of different breeds or color. The differences shown appear to be consistent and, while it is highly improbable that, by chance alone, variations of the same character would be obtained in all of the experiments, the differences indicated are, for the most part, comparatively small and the question arises as to whether they are sufficiently large to be regarded as significant. In order to answer this question, a quantitative comparison of results is necessary.

A general idea of the situation presented may be obtained by a comparison of the mean values for all observations on a given lot of animals, disregarding variations in the magnitude and direction of changes that occurred. A comparison of this kind constitutes the most severe test that can be applied since the occurrence of variations in one direction and then in the other might obliterate any difference between mean values or obscure the effect of an influence which tended to produce a variation in a given direction. On this basis, the results obtained for calcium, inorganic phosphorus, and for the calcium-phosphorus ratio are as follows

*Calcium*

	Group I	Group II	Group III
Control	15 3 ± 09	15 4 ± 09	15 5 ± 10
Light	15 3 ± 07	14 9 ± 07	15 4 ± 08
Dark	15 5 ± 06	15 7 ± 08	15 9 ± 08

*Inorganic Phosphorus*

	Group I	Group II	Group III
Control	4 14 ± 033	4 35 ± 05	4 11 ± 037
Light	4 14 ± 045	4 24 ± 05	3 84 ± 037
Dark	3 98 ± 037	4 10 ± 05	3 94 ± 034

*Calcium-Phosphorus Ratio*

	Group I	Group II	Group III
Control	3 79 ± 04	3 61 ± 05	3 83 ± 04
Light	3 79 ± 05	3 62 ± 05	4 08 ± 04
Dark	3 97 ± 04	3 96 ± 05	4 09 ± 04

These figures show a very close agreement among animals of a given as well as of different groups. In Group I, there is a suggestion that the calcium content of the blood may have been higher in dark than in control or light animals, but in Groups II and III the difference is sufficient to be regarded as significant. In only one case, however, is there a significant difference between the values obtained for light and control animals (Group II). It is also of interest to note that while there is no definitely significant difference between the values for the 3 groups of control animals, there is a difference in the case of both the light and dark divisions of the 3 groups.

Comparison of the results for inorganic phosphorus and for the calcium-phosphorus ratio bring out a similar situation. In general, the value for inorganic phosphorus of animals in the dark is smaller than that for control or light animals, while the calcium-phosphorus ratio is larger. The only exception to this rule is in the results for inorganic phosphorus of light and dark animals in Group III and this may be a result of the change of environment, as suggested by the reversal of relations. Some of the effects indicated by the smooth curves are, therefore, borne out by a quantitative comparison of aggregate mean values. If a comparison is made of values obtained for successive phases of the reaction, differences are found between all classes of animals, and in some instances, they are even more clearly defined than those shown by aggregate mean values.

This does not prove, however, that the differences found are due to light effects. It is possible, but improbable, that by chance selection, the animals were distributed in such a way as to form groups with differences in the basic levels of calcium and inorganic phosphorus which were the same in all cases and that these differences were maintained with some variation throughout the period of observation. It is difficult to eliminate this possibility as there is no entirely satisfactory method of determining basic levels for the several groups of animals due to the disturbance of equilibrium following the change from outdoor to indoor life. This aspect of the problem may be clarified, however, by a comparison of results based on the relations shown by light and dark animals for different periods of the experiments. An examination of the figures given in Table I shows that up

to Nov. 19 the results for calcium on Group I were irregular with no clearly defined difference in levels. From Nov. 19 to Mar. 11, the general level of all values increased but not exactly to the same extent, while from Mar. 25 to May 18, there was a further increase among control and light animals but a decrease occurred in the dark division. In Group II, the differences for successive periods are not so clearly defined, but for comparative purposes the same divisions may be made. In the case of Group III, it will be necessary to adhere to the divisions made by experimental conditions. In this instance, nothing is known concerning basic levels of control, light, or dark animals. The first set of values corresponds with the values for the second period as given for Groups I and II, while the second and third groups of results represent effects produced by a change of environmental conditions. On this basis the mean values for calcium are as follows:

*Group I.*

	Oct. 8 to Nov. 12	Nov. 19 to Mar. 11	Mar. 25 to May 18
Control.....	14.91 $\pm$ .277	15.58 $\pm$ .080	16.04 $\pm$ .133
Light.....	14.59 $\pm$ .179	15.47 $\pm$ .067	15.79 $\pm$ .136
Dark.....	14.77 $\pm$ .106	15.83 $\pm$ .081	15.38 $\pm$ .134

*Group II.*

Control.....	14.42 $\pm$ .151	15.47 $\pm$ .091	15.87 $\pm$ .173
Light.....	14.09 $\pm$ .156	15.14 $\pm$ .080	15.20 $\pm$ .112
Dark.....	15.17 $\pm$ .235	15.87 $\pm$ .074	15.85 $\pm$ .124

*Group III.*

Control.....	15.5 $\pm$ .196	15.3 $\pm$ .084	16.1 $\pm$ .142
Light.....	15.4 $\pm$ .241	15.0 $\pm$ .069	16.1 $\pm$ .142
Dark.....	16.6 $\pm$ .226	15.4 $\pm$ .063	16.1 $\pm$ .134

On the same basis, the divisions that would be made in the results for inorganic phosphorus do not fall at exactly the same points and there is again a difference between Groups I and II. According to the

divisions indicated in Tables II and IV, the comparative results for inorganic phosphorus are as follows:

*Group I.*

	1	2	3
Control	4 42 ± 057	4 07 ± 052	3 83 ± 041
Light	4 51 ± 058	4 01 ± 063	3 64 ± 043
Dark	4 30 ± 036	4 15 ± 052	3 45 ± 044

*Group II.*

	1	2	3	4
Control	4 95 ± 120	4 83 ± 078	4 45 ± 072	3 75 ± 048
Light	4 58 ± 048	4 79 ± 083	4 04 ± 092	3 75 ± 041
Dark	4 80 ± 155	4 47 ± 070	4 04 ± 062	3 55 ± 051

*Group III*

	1	2	3
Control	4 48 ± 083	4 05 ± 044	3 87 ± 056
Light	4 35 ± 085	3 74 ± 037	3 55 ± 056
Dark	3 91 ± 080	4 01 ± 044	3 82 ± 066

Ratio values might be compared in the same way but the results for calcium and phosphorus are sufficient to bring out the points of chief importance. In the first place, it is evident that the relative order of magnitude of the values obtained for calcium and phosphorus cannot be attributed to inherent differences in the basic levels of control, light, and dark animals. It is also evident that definite changes of level occurred in all classes of animals which involved changes in the relative magnitude of values from one period to another. This shows not only an appreciable degree of independence of action of factors responsible for these changes, but that the effect produced was influenced by the length of time animals were exposed to the action of a particular influence. The time influence is best illustrated by the results for dark animals of Group I which showed a clearly defined increase in calcium over a long period of time and a terminal decrease,



while for corresponding periods, inorganic phosphorus was maintained at a higher level than in control or light animals but eventually dropped to a much lower level.

With reference to the effects produced by a change of environment, as in Group III, it may be pointed out that the marked decrease in calcium with a slight, but apparently definite, increase in phosphorus shown by dark animals when placed in the light is conclusive evidence in itself that the first change of environment produced an effect. This result is supported, however, by the occurrence of a marked decrease in phosphorus in the light division. But, calcium did not increase as might have been expected or the increase came too late to affect the mean value for that period. This, in itself, is of importance as it suggests a difference in the rate of response or force of action of positive and negative influences following a prolonged exposure to an influence of the opposite kind.

From a quantitative analysis of the results, one may conclude that, in general, the conditions shown by the smoothed curves give a fair conception of the actual influence of environmental conditions on blood calcium and inorganic phosphorus. But, in attempting to define the action of this influence, it should be pointed out that there is evidence to show that light environment was not the only factor concerned in the production of the variations that occurred and, hence, that the influence exerted by light was subject to modification by other factors. Moreover, it appears that the effect produced in a given case depended to some extent upon the existing levels of calcium and phosphorus as well as the concentration of other substances in the blood which influenced the calcium and phosphorus content or equilibrium. Time is also an important factor as the effect produced varied with the length of exposure to a given influence.

The results obtained show conclusively that both absolute amounts of calcium and inorganic phosphorus in the blood of rabbits and the calcium-phosphorus equilibrium were affected by conditions of light environment which included no short wave-length ultra-violet. A distinction is to be made between effects produced by a change of environment and effects produced by prolonged exposure to a fixed environmental condition of a given nature.

With animals that had not been subjected to prolonged exposure

to a fixed environment, a change of environment produced a more or less marked disturbance of the calcium-phosphorus equilibrium extending over a period of 7 to 9 weeks. On the whole, the effect produced by a change from outdoor to indoor life was greater than that produced by a change from the diffuse light of the laboratory to neon light or darkness. The outstanding features of the effects produced by a change from outdoor to indoor life were first, an increase, and then a decrease in the calcium content of the blood, with changes in inorganic phosphorus in the opposite direction, so that the ratio of calcium to phosphorus was greatly increased and then diminished. These effects were modified by exposure to neon light and by exclusion of light. A change of environment after prolonged exposure to a fixed environment produced only a slight and transient disturbance of equilibrium with a marked delay in the development of effects characteristic of the new environment.

The initial effects of exposure to a fixed environment of neon light or darkness are uncertain. The first definite effect of the exclusion of light on the calcium content of the blood was an increase with the maintenance of a level in the upper ranges of normal; the effect of neon light was a decrease with the maintenance of a level in the lower ranges of normal. Both conditions produced a decrease of inorganic phosphorus, but the decrease was less among animals exposed to neon light and there was evidence of a tendency to maintain a higher level or to prevent a decrease to an extremely low level. The effect of neon light was maintained throughout the period of observation, subject to the modifying influence of other factors, but the early effect of exclusion of light lasted for only a few months; the terminal effect was a decrease of both calcium and inorganic phosphorus.

With the increase in calcium and the decrease in inorganic phosphorus there was a marked increase in the calcium-phosphorus ratio of animals in the dark and this increase continued until near the end of the experiments despite the occurrence of a decrease in calcium. The calcium-phosphorus ratio of animals exposed to neon light increased less and here again there was an obvious tendency to maintain a more stable equilibrium and to prevent the development of an excessively high ratio of calcium to inorganic phosphorus.

The results obtained for animals living in the diffuse light of the

laboratory differed from those of animals living under fixed environmental conditions in several significant respects. They showed wider variations within a given period of time and more frequent fluctuations; calcium tended to be higher than for animals exposed to neon light but lower than for animals in the dark, while inorganic phosphorus was, for the most part, higher than in either of the other groups, but at times was definitely lower. The calcium-phosphorus ratio was very irregular, at times it was much higher than that of other animals, and at other times much lower, due to the occurrence of wide variations from a general level which was somewhat lower than that of animals exposed to neon light. The outstanding features of the condition presented by these animals, as compared with animals living under fixed environmental conditions, was, therefore, the instability of the calcium-phosphorus equilibrium.

While the results have not been analyzed in detail from the point of view of color or breed, sufficient evidence has been presented to show that the reaction to environmental conditions or susceptibility to the light influence is not the same for all classes of animals, but it is uncertain whether this difference is attributable to color or to some more fundamental difference in the constitution of the animal organism.

The data have also been analyzed from the standpoint of the distribution of individual values, but since this form of analysis reveals essentially the same differences as have been noted above, the results are omitted.

The features of the results obtained in these experiments that are of especial interest from the point of view of their bearing on the reaction of the animal organism to environmental conditions are the profound effects produced by a change of light environment and the relation of the effects produced by exposure to light and by exclusion of light to conditions that occur normally as a function of age. The depression or suspension of growth activity and the occurrence of certain changes in organic constitution, associated with an increase in blood calcium and a decrease in inorganic phosphorus, with a marked increase in the calcium-phosphorus ratio produced by deprivation of light are conditions analogous to the changes that occur with advancing age. The effects produced by neon light, on the other hand, suggest that its

action tended to prevent the occurrence of premature aging or to maintain conditions that are compatible with vigorous functional activity. Moreover, available evidence seems to indicate that this analogy between the action of light and changes that occur with age is equally applicable to the effects produced by light environment on susceptibility and the reaction to disease.

In conclusion, it may be said that the results of this series of experiments, including those on nutrition and growth and on organic constitution, show that, under appropriate conditions, it is highly probable that light of any kind is capable of producing a biological effect even though the energy involved may be extremely small.

#### SUMMARY.

A series of experiments was carried out for the purpose of determining whether a light environment comprising radiations of comparatively long wave-length and only a small amount of energy was capable of affecting the chemical equilibrium of the blood as indicated by the calcium and inorganic phosphorus content of the blood of normal rabbits. A study was made of effects produced by prolonged exposure to fixed environmental conditions (neon light and darkness) as compared with a varying environment of diffuse, filtered sunlight and by a change from one environment to another.

It was found that the chemical equilibrium of the blood was definitely affected by the conditions employed and that the effects produced could be correlated with differences in organic constitution on the one hand, and on the other, with certain differences in the functional activity of the same animals, involving nutrition and growth and the proliferative activity of hair follicles.

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## ON CEREBRONIC ACID. VI.

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In a recent paper Klenk<sup>1</sup> has emphatically refuted the accepted view of the structure of cerebronic acid as  $C_{26}H_{50}O_3$ . This statement seems very surprising in the light of the experience of these laboratories. Three different workers, each at a different time, have obtained lignoceric acid on oxidation of cerebronic acid. In the experiments reported by Levene and Taylor<sup>2</sup> the yield of lignoceric acid on oxidation of cerebronic acid was 81 per cent of the theory. A yield of this proportion could not be attributed to an impurity. Furthermore, the lignoceric acid obtained from the cerebronic acid was converted into the corresponding pentacosanic acid, which had the correct molecular weight, and the latter acid again was converted into a hydroxypentacosanic acid, which had the correct molecular weight. It is hard to conceive how Klenk considers his evidence more convincing than that furnished by Levene and West<sup>3</sup> and by Levene and Taylor.<sup>2</sup> However, in view of the very emphatic assertions of Klenk, we concluded to repeat the oxidation of cerebronic acid under more rigorous conditions than in the experiments of Levene and Taylor. Nevertheless, the main product of oxidation was an acid of the composition  $C_{24}H_{48}O_2$ , having the molecular weight of 369 (the theory requiring 368) and a melting point of 79.0-80.5°. Thus we shall adhere to the older view of the composition of cerebronic acid until Klenk shall have furnished more convincing evidence in favor of his theory. We may add, however, that we have some reason to believe

<sup>1</sup> Klenk, E., *Z. physiol. Chem.*, 1928, clxxiv, 214.

<sup>2</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.

<sup>3</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913-14, xvi, 475.

that the acid  $C_{23}H_{46}O_2$  of Klenk was a mixture of two acids, one of  $C_{24}$  and the other of  $C_{22}$ . A pure sample of the acid  $C_{23}H_{46}O_2$  was obtained by us in an experiment in which a larger sample of cerebronic acid was oxidized by the usual method. The cerebronic acid in that experiment had a melting point of  $99.5\text{--}100.5^\circ$  and a specific rotation of  $[\alpha]_D = +3.7^\circ$ .

#### EXPERIMENTAL.

*Preparation of Phrenosin.*—Mixed crude cerebrosides which had been kept in the laboratory for several years were purified in the following way. First, they were recrystallized several times from glacial acetic acid and this product was then recrystallized a few times from pyridine. The material was freed from pyridine by extraction with acetone. The dry residue was then recrystallized several times from a solution of equal parts (by volume) of methyl alcohol and of chloroform until further recrystallization no longer affected the specific rotation of the phrenosin. The rotation of this material in pyridine was  $[\alpha]_D^{20} = +5.0^\circ$ .

*Cerebronic Acid.*—The cerebronic acid was obtained either by hydrolysis in an autoclave for 24 hours, or by alcoholysis by means of alcohol containing 8 per cent of sulfuric acid. The acid was purified by conversion into the lead salt and subsequent removal of the lead ion. Often the operation had to be repeated several times. The purified material melted at  $130^\circ$  and gave the following analysis:

4.355 mg. substance: 12.040 mg.  $CO_2$  and 4.970 mg.  $H_2O$ .

0.500 gm. " required 12.55 cc. of 0.1 N NaOH.

$C_{23}H_{46}O_2$ . Calculated. C 75.33, H 12.50, mol. wt. 398.

Found. " 75.39, " 12.76, " " 398.5

The molecular weight was 398.5 and the optical rotation in pyridine was

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 3.0} = +4.66^\circ.$$

*Oxidation of Cerebronic Acid.*—Of the cerebronic acid just described, 24 gm. were oxidized in acetone solution with potassium permanganate, an excess of permanganate being employed. The oxidation was carried out in three lots, practically in the same manner as described

by Levene and Taylor, with the slight modification that each sample of 8.0 gm. of the acid was oxidized in 1000 cc. of acetone containing 10.0 gm. of potassium permanganate. By extracting the manganese dioxide with hot methyl alcohol the salts of the fatty acids were obtained, which yielded 14.0 gm. of fatty acids. These were fractionated in the manner described in the previous paper. Two fractions were obtained. The lithium salts were converted into the free acids, which were recrystallized from acetone. The larger fraction in this state of purity had the following composition.

4.140 mg. substance: 11.885 mg.  $\text{CO}_2$  and 4.995 mg.  $\text{H}_2\text{O}$ .  
 $\text{C}_{24}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13.  
Found. " 78.28, " 13.50.

The yield was 9.0 gm. It was then further purified by being passed through the lead salt. The acid then had the following composition.

3.775 mg. substance: 10.805 mg.  $\text{CO}_2$  and 4.470 mg.  $\text{H}_2\text{O}$ .  
0.3000 gm. " required 8.10 cc. of 0.1 N NaOH.  
 $\text{C}_{24}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13, mol. wt. 368.  
Found. " 78.05, " 13.24, " " 370.

The molecular weight was 370. The acid melted (not absolutely sharply) at 79–80°. It was recrystallized several times from acetone and then from ether. The melting point was raised only slightly, 79.0–80.5°, and the molecular weight remained unchanged.

0.300 gm. substance required 8.13 cc. of 0.1 N NaOH. Mol. wt. 369.

The substance was then esterified. 5 gm. of the acid were taken up in 200 cc. of 98.5 per cent alcohol containing 2.5 gm. of sulfuric acid and refluxed overnight. On cooling, the ester settled out in plates. The ester was taken up in ether, washed free of adhering sulfuric acid, and the ester obtained on evaporation of the ether was recrystallized from alcohol. It melted sharply at 56–57°.

The mother liquor from the first crystallization was concentrated to 100 cc. and in the solution a deposit of glistening plates formed. The yield was 1.3 gm. It was dissolved in ether and freed from sulfuric acid. The residue from the ether was recrystallized from alcohol. The substance melted at 79–80°. It had the following composition.

0.0986 gm. substance: 0.2824 gm.  $\text{CO}_2$  and 0.1156 gm.  $\text{H}_2\text{O}$ .  
 0.2000 " " required for neutralization 5.45 cc. of 0.1 N NaOH.  
 $\text{C}_{26}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13, mol. wt. 368.  
 Found. " 78.10, " 13.22, " " 367.

Hence this substance was lignoceric acid.

The ester melting at  $56-57^\circ$  was distilled. It distilled at  $205-210^\circ$ , leaving only a small residue. The distillate melted at  $56-57^\circ$ . The ester was converted into the free acid in the usual way. The acid, after recrystallization from benzene, melted at  $78-79^\circ$  and solidified at  $76^\circ$ . It had the following composition.

0.0986 gm. substance: 0.2830 gm.  $\text{CO}_2$  and 0.1156 gm.  $\text{H}_2\text{O}$ .  
 0.2000 " " required for neutralization 5.43 cc. 0.1 N NaOH.  
 $\text{C}_{26}\text{H}_{48}\text{O}_2$ . Calculated. C 78.15, H 13.13, mol. wt. 368.  
 Found. " 78.26, " 13.22, " " 368.3.

The lower melting points may be due to the fact that the cerebronic acid was obtained from cerebrosides prepared many years ago, or perhaps to slight admixtures of the lower acids to be described in another paper.



## OXIDATION OF LIGNOCERIC ACID.

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Recently<sup>1</sup> in the oxidation of a sample of cerebronic acid to the next lower unsubstituted fatty acid, considerable difficulty was experienced in obtaining a pure substance. Instead of pure lignoceric acid, a product was isolated that appeared to be a mixture of acids. It became of interest then, to determine whether an  $\alpha$ -hydroxy acid of known purity related structurally to cerebronic acid could be degraded by oxidation, under conditions similar to those applied to cerebronic acid, to the next lower acid of the series in good yield and uncontaminated by substances that could not be separated easily.

Lignoceric acid was therefore converted into its next lower homologue by passing it through the following steps:<sup>2</sup>



These reactions had been partly carried out by Meyer, Brod, and Soyka<sup>3</sup> in 1913 in their preparation of the docosanoic acid related to lignoceric acid and by Levene and Taylor<sup>4</sup> in 1922. Since the work of Levene and Taylor, it has become known<sup>5</sup> that some higher aliphatic substances are changed on standing so that it is very difficult to bring them back to their original melting points. The materials used by Levene and Taylor having stood for some time, it was desirable to check their figures.

The present experiments show that the melting points recorded both by Meyer, Brod, and Soyka and by Levene and Taylor should be

<sup>1</sup> Unpublished experiments.

<sup>2</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913-14, xvi, 475.

<sup>3</sup> Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxiv, 1113.

<sup>4</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1922, lii, 227.

<sup>5</sup> Levene, P. A., and Taylor, F. A., *J. Biol. Chem.*, 1924, lix, 905.

revised upward. A comparison is given, in Table I, of the various determinations.

It is also quite evident that the oxidation of  $\alpha$ -hydroxylignoceric acid gives rise to a single substance, isotricosanoic acid, since extended attempts at purification failed to bring about a significant change in properties. The melting point of the crude acid was 75–77° while the best specimens, obtained by allowing very small fractions to separate from ether, melted at 76.5–77.5°.

The melting point of the isotricosanoic acid brings out an interesting relationship in the lignoceric series. Whereas in the normal acids the curve of melting points of the acids with odd numbers of carbon atoms is considerably below that of the even numbered acids, the curves in the lignoceric series are apparently almost superimposed. In the neighborhood of C<sub>23</sub>–C<sub>24</sub>, in the normal series<sup>4</sup> the odd numbered acids melt at a temperature approximately 1° lower than the preceding

TABLE I.

	Meyer, Brod, and Soyka	Levene and Taylor	Taylor and Levene
$\alpha$ -Bromolignoceric acid	68–69°	68 5°	69 5–70 5°
$\alpha$ -Hydroxylignoceric acid	91–92°	91–92°	94–95°
Isotricosanoic acid ...		73 5°	76 5–77 5°

even numbered acids. Meyer, Brod, and Soyka<sup>3</sup> prepared the docosanoic acid from lignoceric acid. It melted at 75°. In this series then, the odd numbered acid melts at a point between the melting points of the two adjacent even numbered acids. This constitutes further evidence in favor of the view first advanced by Meyer, Brod, and Soyka, that lignoceric acid is not a normal acid.

Recently Klenk<sup>6</sup> has obtained from cerebronic acid, by oxidation with permanganate, a tricosanoic acid which melts at 78.5°. The relationship of that acid to the acid described here may prove of interest.

#### EXPERIMENTAL.

The lignoceric acid was prepared from peanut oil by a method that was essentially that described by Levene, Taylor, and Haller.<sup>7</sup> The

<sup>6</sup> Klenk, E., *Z. physiol. Chem.*, 1928, clxxiv, 214.

<sup>7</sup> Levene, P. A., Taylor, F. A., and Haller, H. L., *J. Biol. Chem.*, 1924, lxi, 157.

mixture of insoluble fatty acids from peanut oil was cooled to 20–22° overnight in centrifuge bottles and centrifuged until there was very little further packing of the sediment. The sediment was then washed repeatedly with 95 per cent alcohol, in the centrifuge bottles, until the supernatant solution was nearly colorless and the solid acids had been reduced in volume to about 10 per cent of the original. Further removal of liquid acids was accomplished by three crystallizations from 95 per cent alcohol at 20–24°. The melting point was then 72.5–73.5° and the yield was about 2.5 per cent of the insoluble fatty acids of the peanut oil. Alcohol then failed to raise the melting point. The acid crystallized in lustrous plates.

For final purification the material was crystallized from pyridine and from ether until the melting point was 80–81° when the bath was so heated, with stirring, that the temperature rose 1° in 7 to 8 seconds. It solidified sharply at 77°.

0.1001 gm. substance: 0.2871 gm. CO<sub>2</sub> and 0.1159 gm. H<sub>2</sub>O.

0.5001 " " required 6.74 ml. 0.2 N NaOH.

C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.26, H 13.04, mol. wt. 368.

Found. " 78.21, " 12.96, " " 371.

This molecular weight, and all others, were obtained by dissolving the acid in a mixture of 50 ml. of methyl alcohol and 25 ml. of toluene and titrating the hot solution with approximately 0.2 N NaOH in the presence of phenolphthalein. A correction of 0.07 ml., the difference between the alkali required to titrate a standard acid potassium phthalate solution in water and in the mixture of methyl alcohol and toluene, respectively, was subtracted. The titrations were carried out with a burette graduated in 0.02 ml. divisions. In this way molecular weights are obtained which, with fatty acids of known purity, are uniformly 2 to 3 units higher than the calculated values.

*α-Bromolignoceric Acid*.—Lignoceric acid (3.1 gm.) was melted on the water bath with red phosphorus (0.2 gm.) and bromine (4.9 gm.) dropped in slowly. The heating was continued for 4 hours, at which time an excess of bromine was still present. The acid bromide was poured into water and stirred until the acid was liberated. It was then collected in ether, dried, and the ether distilled. The residue was crystallized from petroleum ether at 0°. M. p. 67.5–68.5°. The

yield was 90 per cent of the theoretical. Two further crystallizations brought the melting point to 69.5–70.5° after which it could be changed no further. Meyer, Brod, and Soyka<sup>3</sup> as well as Levene and Taylor<sup>4</sup> found a melting point of 68–69°.

0.1859 gm. substance: 0.0763 gm. AgBr (Carius).  
 0.5012 " " required 5.61 ml. 0.2 N NaOH.  
 $C_{24}H_{48}O_2Br$ . Calculated. Br 17.88, mol. wt. 447.  
 Found. " 17.47, " " 447.

*$\alpha$ -Hydroxylignoceric Acid.*—The  $\alpha$ -bromolignoceric acid was heated on the water bath with a large excess of 10 per cent sodium hydroxide solution for 40 hours. The cooled solution was neutralized with hydrochloric acid, the soap filtered off, and the acid liberated in the presence of ether with hydrochloric acid. The ether solution was washed with water, dried, and the ether distilled. The residue was crystallized from acetone at 0° and then melted at 93–94°. The yield was 90 per cent of the theoretical. Meyer, Brod, and Soyka<sup>3</sup> and Levene and Taylor<sup>4</sup> record a melting point of 91–92°. The molecular weight was 388. For further purification the acid was converted into the ethyl ester and crystallized from absolute alcohol. M. p. 57.5–59°. The ester was saponified and the acid liberated as before. It now melted at 94–95°. Continued crystallization and passage over the lead salt failed to alter it further.

0.1001 gm. substance: 0.2762 gm.  $CO_2$  and 0.1131 gm.  $H_2O$ .  
 0.4999 " " required 6.46 ml. 0.2 N NaOH.  
 $C_{24}H_{48}O_2$ . Calculated. C 75.00, H 12.50, mol. wt. 384.  
 Found. " 75.24, " 12.65, " " 387.

*Isotricosanoic Acid.*—The  $\alpha$ -hydroxylignoceric acid was dissolved in boiling acetone and treated with a slight excess of potassium permanganate. The solution was then boiled until the purple color was destroyed. After cooling, the mixture of manganese dioxide and soap was filtered off, dried, and decomposed with sodium bisulfite and hydrochloric acid, the fatty acid being collected in ether. The ether solution was dried and evaporated. The residue crystallized from acetone at 0° in lustrous plates and melted at 75–77°. The molecular weight was 357 and the yield was 80 per cent of that calculated.

The ethyl ester was prepared in the usual manner and crystallized

at 0°. It was recrystallized from alcohol and then melted at 51.2–52.2°. After distillation at 0.1 mm. it was saponified and the acid liberated in the presence of ether as before. After crystallization from acetone, the melting point was 76.5–77.5°. It solidified at 74.5–74°. Passage over the lead salt and crystallization from ether failed to change the melting point. It was finally separated into six fractions by allowing its ether solution to evaporate slowly at 0° and decanting the supernatant solution after each successive small amount of acid had separated. The melting points of these fractions were identical with the last given above. Levene and Taylor<sup>4</sup> record a melting point of 73.5°.

0.1002 gm. substance: 0.2870 gm. CO<sub>2</sub> and 0.1157 gm. H<sub>2</sub>O.

0.5005 “ “ required 6.99 ml. 0.2 N NaOH.

· C<sub>23</sub>H<sub>46</sub>O<sub>2</sub>. Calculated. C 77.97, H 12.99, mol. wt. 354.

Found. “ 78.10, “ 12.93, “ “ 358.

The melting points are corrected.



## HEXOSEDIPHOSPHATE.

BY P. A. LEVENE AND ALBERT L. RAYMOND\*

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(Received for publication, October 19, 1928.)

The problem of the structure of the hexosediphosphate which forms during enzyme fermentations has attracted the attention of many investigators. Lebedev<sup>1</sup> has shown that the osazone, in whose formation one phosphate has been eliminated, gives glucosazone on alkaline hydrolysis; Meyerhof and Lohmann,<sup>2</sup> using Willstätter's iodate method, have found that the diphosphate is almost pure ketose. These two observations have been corroborated by the isolation of fructose after both acid<sup>3</sup> and enzyme<sup>4</sup> hydrolysis of the diphosphate. Therefore although Kluyver and Struyk<sup>5</sup> do not agree, it seems most probable that the diphosphate is a derivative of fructose.

It has been found, moreover, that on preparing the hydrazone of the diphosphate, both phosphate groups are retained, while on forming the osazone one is eliminated.<sup>6</sup> This has been interpreted as indicating that the labile phosphate is attached to carbon atom (1). The argument receives support from work of our own (unpublished) in which it was found that synthetic 1-fructose phosphate, prepared from  $\beta$ -diacetone fructose, behaves identically. Here also the phosphate is retained in the hydrazone and eliminated with the formation of glucosazone.

These results lead to the conclusion that the ester is fructose di-

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<sup>1</sup> Lebedev, A., *Biochem. Z.*, 1910, xxviii, 213.

<sup>2</sup> Meyerhof, O., and Lohmann, K., *Naturwissenschaften*, 1926, xiv, 1277.

<sup>3</sup> Young, W. J., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 528.

<sup>4</sup> Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, 1910, lxxxii, 321.

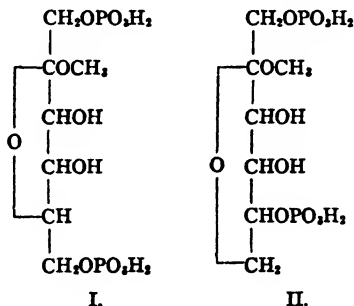
<sup>5</sup> Kluyver, A. J., and Struyk, A. P., *Naturwissenschaften*, 1926, xiv, 882.

<sup>6</sup> Lebedev, A., *Biochem. Z.*, 1910, xxviii, 213; 1911, xxxvi, 248. Young, W. J., *Biochem. Z.*, 1911, xxxii, 178.

phosphate with one phosphate attached to the first carbon atom. With regard to the second phosphate, however, no evidence has been presented and the present paper deals briefly with this phase of the problem.

As has been shown, the glucosides exhibit a quite different behavior on acid hydrolysis depending upon their belonging to the  $<1, 4>$  or  $<1, 5>$  ring form. Similar effects have been found in the case of the fructosides and we thus appear to have a general method of distinguishing the five-membered from the six-membered lactal glucosides.

In the case of the hexosediphosphate glucoside a  $<2, 5>$  ring would be required if the second phosphoric acid radical were attached to carbon atom (6) (Formula I), and would be prevented if it were on carbon atom (5) (Formula II), while the other possible positions of the phosphate would permit either ring form.



Thus by preparing the diphosphate glucoside and subjecting it to hydrolysis with dilute acid we might hope to obtain valuable information as to the allocation of the second phosphate.

Actually the hydrolysis has been done by Morgan<sup>7</sup> in his excellent paper on the diphosphate glucosides but he apparently did not consider its significance and the conditions which he chose were not such as to make the results conclusive as to the lactal structure. It is possible to conclude from his data, however, that the  $\alpha$ - and  $\beta$ -glucosides have the same lactal ring as their rates of hydrolysis are of the same order of magnitude. We have repeated the preparation of the glucoside,

<sup>7</sup> Morgan, W. T. J., *Biochem. J.*, 1927, **xxi**, 675.



modifying Morgan's procedure slightly, and have subjected it to hydrolysis with 0.1 *N* hydrochloric acid in a steam bath. Under these conditions the  $\gamma$ -glucosides are very rapidly hydrolyzed while normal derivatives show only slight hydrolysis.

In the case of the diphosphate it was found that the reduction increased very rapidly at first (Fig. 1) and then quite slowly for the

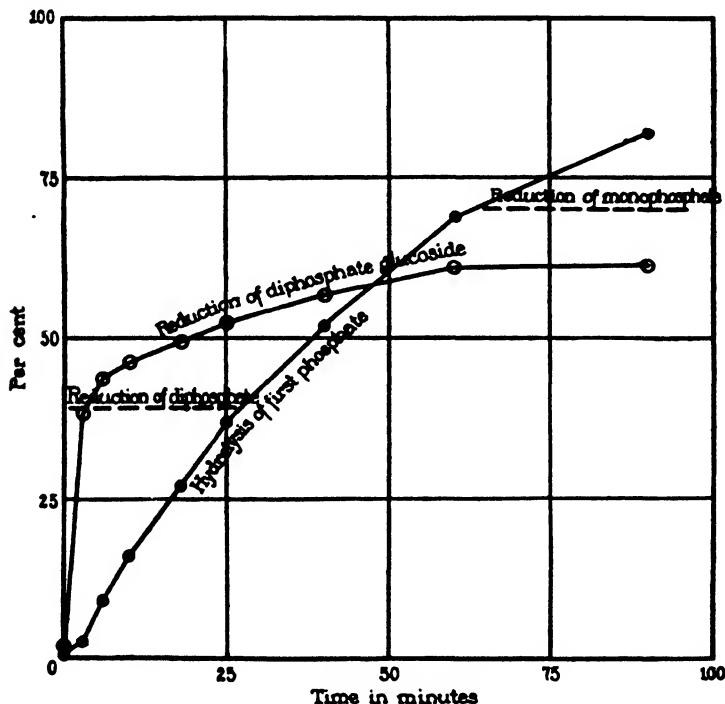
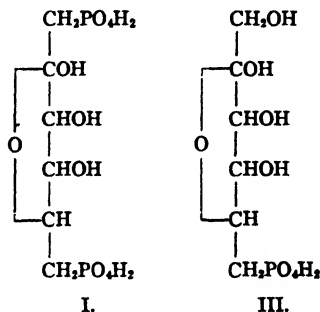


FIG. 1.

duration of the experiment (90 minutes). The latter slow increase was apparently due to the hydrolysis of the diphosphate to monophosphate, as was shown by the increase in inorganic phosphate, while the first rapid increase represented the hydrolysis of the glucoside to form the diphosphate. This view was confirmed by the fact that the reduction of the diphosphate corresponded to the first change as is

seen in the figure, while the final reduction value approached that of the monophosphate as a limit.

The very rapid hydrolysis of the glucoside makes it reasonably certain that we are dealing with a  $\gamma$  derivative and this in turn, from what has been said before, leads to the conclusion that the second stable phosphate is on carbon atom (6). The hexosediphosphate therefore has the structure (I) while the Neuberg ester must have the corresponding structure (III).



#### EXPERIMENTAL.

*1. Preparation of Hexosediphosphate Glucoside.*—Candiolin<sup>6</sup> was converted to the sodium salt and then to the barium salt and the latter was very thoroughly dried. 100 gm. of the dry salt were dissolved in 2 liters of dry methyl alcohol containing 34 gm. of dry hydrogen chloride gas as described by Morgan.<sup>7</sup> After standing 24 hours at room temperature the solution was neutralized by passing in dry ammonia gas until faintly alkaline to moist litmus paper. The barium salt which precipitated was filtered with suction, washed with methyl alcohol, and dried *in vacuo*. The dry salt was powdered and repeatedly extracted with water. The aqueous extracts were concentrated under reduced pressure and the pH adjusted to 9.0. An equal volume of 95 per cent alcohol was added with stirring and the salt thus precipitated was purified by repeated solution and precipitation.

<sup>6</sup> We wish again to thank the Winthrop Chemical Company, Inc., for giving us this material.

*Analysis.*

0.0937 gm. substance:	0.0658 gm. BaSO <sub>4</sub> .
0.0937 " " :	0.0312 " Mg <sub>3</sub> P <sub>2</sub> O <sub>7</sub> .
0.1462 " " :	0.0556 " AgI.
C <sub>7</sub> H <sub>12</sub> O <sub>12</sub> P <sub>3</sub> Ba <sub>2</sub> . Calculated.	Ba 43.96, P 9.94, OCH <sub>3</sub> 4.96.
Found.	" 41.32, " 9.27, " 5.02.

2. *Hydrolysis*.—1.0 gm. (dry 0.94 gm.) of the barium salt prepared as above was dissolved in water, the barium was quantitatively precipitated with sulfuric acid, and the mixture was diluted to 21.0 cc., and centrifuged. The solution was filtered to remove a slight turbidity and cooled in ice water. To 18.0 cc. were added 5.2 cc. of ice-cold 0.45 N hydrochloric acid. The final solution contained 0.1 N hydrochloric acid and 0.055 molal ester (equivalent to 10 mg. of hexose per cc.).

Into small Pyrex test-tubes were pipetted 2.5 cc. of the above solution. These were frozen in a solid CO<sub>2</sub>-alcohol bath and were kept frozen in an ice-salt mixture. The tubes were sealed in a blast lamp and replaced in the ice-salt bath. These operations were all performed as rapidly as possible to prevent hydrolysis.

The sealed tubes, with one exception, were removed from the ice-salt and transferred to a boiling water bath. At measured time intervals a tube was removed, shaken well, immediately refrozen in the alcohol-solid CO<sub>2</sub> and replaced in the ice-salt mixture. As soon as possible the tubes were opened and the contents allowed to melt. Samples were then removed for reduction and inorganic phosphate determinations which were done immediately.

3. *Analytical*.—The phosphate determinations were done by the method of Kuttner-Cohen modified as previously described.

For the reduction determinations the Lehmann-Maquenne<sup>9</sup> technique was employed but as the material was limited in amount, the method was modified by using only one-tenth as much of each reagent as indicated in the usual procedure.<sup>10</sup> The results were quite as satisfactory for our purpose as when the larger quantities were employed although the accuracy was somewhat less.

The reduction was also determined on solutions of the mono- and

<sup>9</sup> Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, 1928, lxxix, 621.

<sup>10</sup> See Griesbach, W., and Strassner, H., *Z. physiol. Chem.*, 1913, lxxxviii, 199.

diphosphates which were prepared by removing the barium from known quantities of the pure barium salts with sulfuric acid. The monophosphate had been prepared by hydrolysis of the diphosphate and purified by repeated precipitation of the barium salt from aqueous solution with alcohol. The diphosphate had been purified by recrystallizing its strychnine salt and then converting to the barium salt.

In order to standardize the results with the technique employed, the reduction was determined for a pure sample of fructose and the analyses were corrected by the factor thus found. In Fig. 1 the reduction is expressed in per cent and plotted as a function of the time of hydrolysis. In making the calculation the theoretical reduction corresponding to complete hydrolysis to fructose was taken as standard. The reductions of the mono- and diphosphates similarly expressed, are included for comparison. Also in the same figure, there is given the change in inorganic phosphate, expressed as percentage of the first phosphate hydrolyzed at a particular time.

#### SUMMARY.

On the basis of the rate of hydrolysis of the methyl glucoside of the fructose diphosphate of fermentation, it is concluded that the substance has the  $<2, 5>$  lactal structure.

From this it follows that the stable phosphoric acid residue is attached to carbon atom (6) and also that Neuberg's monophosphate is 6-fructose phosphate.

## ON INOSINIC ACID.

### IV. THE STRUCTURE OF THE RIBOPHOSPHORIC ACID.

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(Received for publication, November 19, 1928.)

There occur in nature ribonucleotides of two types. One is represented by inosinic acid, a ribonucleotide of animal origin, and the other is represented by adenylic and guanylic acids, which are components of yeast nucleic acid and thus are of plant origin. The principal distinction between the two types of nucleotides lies in the rate of hydrolysis of the phosphoric acid from the nucleosides. Yamagawa,<sup>1</sup> working in this laboratory, found the ratio between the constants of hydrolysis  $K$  (inos.):  $K$  (aden.) = 1:3.5. It was assumed that these differences are due to the differences in allocation of the phosphoric acid radicle. Levene and Jacobs<sup>2</sup> had previously concluded that in inosinic acid the phosphoric acid radicle is attached to carbon atom (5). This conclusion was reached on the basis of the result of oxidation of the phosphoribose obtained from inosinic acid. Under these conditions phosphoribonic and not phosphotrihydroxyglutaric acid was obtained. Recently Professor Robinson<sup>3</sup> has expressed the idea that ribose does not exist performed in the nucleotides, but that it is formed during the process of hydrolysis through a Walden inversion. The assumption of Robinson presupposes the allocation of the phosphoric acid to one of the asymmetric carbon atoms, hence not to carbon atom (5). In view of this suggestion it seemed desirable to test the conclusion of Levene and Jacobs by still another method. Levene and Simms<sup>4</sup> have shown that from the curve of

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<sup>1</sup> Yamagawa, M., *J. Biol. Chem.*, **43**, 339 (1920).

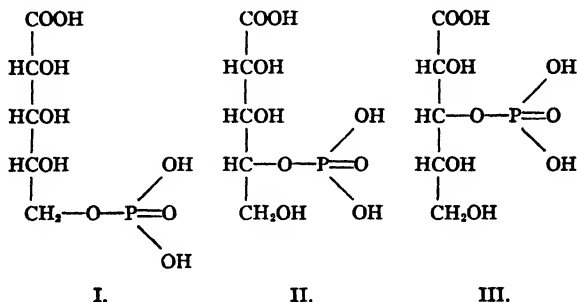
<sup>2</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 746 (1911).

<sup>3</sup> Robinson, R., *Nature*, **120**, **44**, 656 (1927). Levene, P. A., *Nature*, **120**, 621 (1927).

<sup>4</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **65**, 31 (1925).

lactone formation of a sugar acid it is possible to differentiate between  $<1,4>$  and  $<1,5>$  lactones. In a publication of Levene and Wolfrom<sup>5</sup> a plan was developed by which the allocation of a substituting group can be determined on the basis of the curve of lactone formation of a substituted sugar acid.

In the case of phosphoribonic acid the possibilities are the following.



(1) The phosphoric acid radicle is attached to carbon atom (5) as in formula (I). In this case a  $<1,4>$  lactone will be formed; the course of its formation will be slow and the equilibrium will be established only after several days.

(2) The acid has the structure indicated in formula (II). A  $<1,5>$  lactone will be formed; the curve of the progress of lactone formation will show a rapid rise and equilibrium will be reached in less than 2 hours.

(3) The phosphoric acid radicle may be attached in position (3) as given in formula (III) or in position (2). In this case both the  $<1,4>$  and the  $<1,5>$  lactones can form; therefore, the curve of lactone formation will have the character of that of mannonic lactone formation.

The phosphoribonic acid used in the present experiments was prepared by two methods, one sample by oxidation with nitric acid and the second by oxidation with barium hypoiodite, according to the method of Willstätter and Schudel as modified by Goebel.<sup>6</sup> As in the earlier experiments of Levene and Jacobs, the acid was obtained

<sup>5</sup> Levene, P. A., and Wolfrom, M. L., *J. Biol. Chem.*, **77**, 671 (1928).

<sup>6</sup> Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

in the form of the neutral calcium salt having the composition  $(C_6H_9O_9P)_2Ca_3$ . In order to avoid a high hydrogen ion concentration of the solution, the monobasic salt of the composition  $(C_6H_{10}O_9P)_2Ca$  was prepared. A solution of this salt was obtained by adding 2 equivalents of hydrochloric acid to the solution of the neutral salt.

TABLE I.  
*Changes in Rotation of 0.0825 N Monocalcium Phosphoribonate Solution*  
 $l = 2dm.$   $t = 25^\circ$

Time	$\alpha_D^{25}$	$[\alpha]_D^{25}$
<i>min</i>	<i>degrees</i>	<i>degrees</i>
15	-1 11	-12 68
30	-1 10	-12 58
45	-1 10	-12 58
<i>hrs</i>		
1	-1 10	-12 58
2	-1 07	-12 23
3	-1 04	-11 89
4	-1 02	-11 66
6	-0 99	-11 32
8	-0 95	-10 86
10	-0 92	-10 52
24	-0 77	-8 80
32	-0 67	-7 66
48	-0 49	-5 60
56	-0 44	-5 03
72	-0 34	-3 89
81	-0 30	-3 43
98	-0 21	-2 40
103	-0 19	-2 17
120	-0 13	-1 49
129	-0 11	-1 26
146	-0 08	-0 91
168	-0 07	-0 80
192	-0 07	-0 80

The progress of lactone formation is given in Table I and from this table it is seen that the lactone formation proceeds very gradually, reaching an equilibrium in 150 hours. From the character of the curve it follows, then, that the phosphoribonic acid obtained from inosinic acid has the phosphoric acid in position (5), as given in formula (I).

## EXPERIMENTAL.

Barium inosinate was prepared from fish meat extract<sup>7</sup> in the usual way. This was hydrolyzed by boiling with 1 per cent hydrochloric acid, and the barium ribophosphate was isolated according to the directions of Levene and Jacobs.<sup>2</sup>

The barium ribophosphate was then oxidized to phosphoribonic acid in two different ways.

First, the barium salt was treated with barium hypiodite by the general method of Willstätter and Schudel as modified by Goebel.<sup>6</sup>

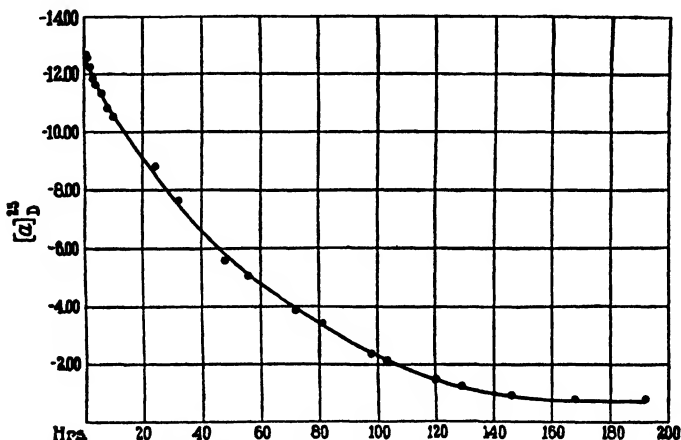


FIG. 1. Mutarotation of ribophosphoric acid.

Second, the free ribophosphoric acid prepared from the barium salt was oxidized with nitric acid by exactly the same procedure as that described by Levene and Jacobs.<sup>2</sup>

In both cases the phosphoribonic acid thus obtained was isolated as its calcium salt. The products were shown by analysis and by determination of their rotations to be identical. The substance analyzed as follows.

<sup>7</sup> We are indebted to Professor Yamagawa, Tokyo Imperial University, for the fish meat extract used in this work.



5.265 mg. substance: 35,450 mg. ammonium phosphomolybdate.

0.1000 gm. " : 0.0272 gm. CaO.

$(C_6H_5O_4P)_3Ca_2$ . Calculated. P 10.23, Ca 19.81.

Found. " 9.77, " 19.44.

For the mutarotation experiment, 0.2500 gm. of the dried substance was treated with 2 equivalents of hydrochloric acid and the volume was made up to 5 cc. The optical rotation was observed in a 2 dm. tube at 25°. The results are shown in Table I and Fig. 1.



## THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

### IV. ENZYME HYDROLYSIS OF DIPEPTIDES.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 20, 1928.)

In previous publications on this subject by Levene, Simms, and Pfaltz<sup>1</sup> and by Levene and Simms<sup>2</sup> the conclusion was reached that the rate of hydrolysis of di- and tripeptides is a function of the intrinsic dissociation constants of the two groups involved in the peptide linkage. A comparison was made between peptides formed from amino acids with free amino groups and peptides formed from amino acids methylated on the nitrogen atom. These series were selected for the reason that the dissociation constants of the two types of substances differ markedly. The progress of hydrolysis in the methylated peptides was complicated by the great tendency towards keto-piperazine formation. If this complicating circumstance had not existed, that is, if the rate of hydrolysis of unsubstituted peptides had been measured, the reaction should have been found to follow the course of an uncomplicated monomolecular reaction. To test the correctness of this conclusion, several peptides of this series have been prepared and have been subjected to the action of erepsin. The erepsin solution used in the older experiments was prepared from intestinal juice;<sup>3</sup> in the present experiments a more active enzyme solution was prepared according to directions of Northrop.

The results of our observations are summarized in Table I. From these results the following conclusions are warranted.

<sup>1</sup> Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924); **70**, 253 (1926).

<sup>2</sup> Levene, P. A., and Simms, H. S. *J. Biol. Chem.*, **62**, 711 (1924-25).

<sup>3</sup> The intestinal juice was obtained from the Battle Creek Sanitarium through the kindness of Dr. Kellogg and Professor Boldyreff.

1. The reactions, under the conditions employed by us, follow the course of a monomolecular reaction.

2. In concentrations of substrate varying from 0.025 M to 0.500 M the rate of hydrolysis is nearly directly proportional to the concentration of enzyme. When a peptide composed of glycine and a *d,l*-acid is compared with one composed of glycine and a naturally occurring amino acid in regard to their rates of hydrolysis, the concentrations of the two substances should be in the ratio of 2:1; when one

TABLE I.  
*Hydrolysis Constants of Peptides.*

	Concentration	$k \cdot 10^4$
	M	
Glycyl-glycine	0 025	74
"	0 050	47
"	0 100	26
"	0 500	4
" + 2 equivalents glycine	0 050	22
Glycyl- <i>d,l</i> -alanine	0 100	87
"	0 200	40
Glycyl-dextro-valine	0 100	22
Glycyl-levo-valine <sup>4</sup>	0 100	0
Glycyl-dextro-isovaline	0 100	0
Glycyl-levo-leucine	0 100	33
<i>d,l</i> -Alanyl-glycine <sup>5</sup>	0 100	150
"	0 200	60
Levo-alanyl-glycine <sup>4</sup>	0 100	0
<i>d,l</i> -Alanyl- <i>d,l</i> -alanine	0 100	8
Dextro-alanyl-dextro alanine	0 100	150
Levo-alanyl-dextro-alanine <sup>4</sup>	0 100	0

peptide is composed of two *d,l*-acids and the other of two optically active naturally occurring acids, the concentrations should be in the ratio of 4:1.

<sup>4</sup> The very slow increase in the titer of these solutions is probably due to the presence of small quantities of the enantiomorphous peptides of the naturally occurring amino acids. Partial racemization may also occur (see foot-note 5).

<sup>5</sup> It should be noted in the case of *d,l*-alanyl-glycine (Table V, Column 3) that after the theoretical value of 3 00 cc. (representing complete hydrolysis of dextro-alanyl-glycine) has been reached, there is a very slow increase in the titer of the solution. This phenomenon may be explained by the assumption that the levo-alanyl-glycine, which remains unattacked by erepsin, is slowly racemized, either by the weak alkali or by an enzyme; the dextro-alanyl-glycine resulting from this partial racemization is then hydrolyzed by erepsin.

3. The rates of hydrolysis at the same concentration of glycylglycine, glycyl-dextro-valine, and glycyl-levo-leucine are of the same order of magnitude. This behavior was expected on the basis of previous experiments.

4. The behavior of peptides containing dextro-alanine differs from that of the peptides enumerated in (3). The highest rate of hydrolysis is observed in the case of dextro-alanyl-dextro-alanine; next in order is *d,l*-alanyl-glycine; the lowest rate is found for glycyl-*d,l*-alanine. A definite explanation of the exceptional behavior of alanine-containing peptides cannot be given at this stage of the work.

5. The presence of free glycine retards the hydrolysis of glycyl-glycine.

6. Peptides containing one amino acid enantiomorphous to that occurring naturally are not hydrolyzed by erepsin. This observation is in harmony with those of Abderhalden. On digestion of *d,l*-alanyl-glycine and similarly constituted peptides, the form containing the amino acid enantiomorphous to that occurring naturally remains intact.<sup>5</sup>

7. Glycyl-dextro-isovaline is not hydrolyzed by erepsin, although dextro-isovaline is configurationally related to the amino acids occurring in proteins.<sup>6</sup>

#### EXPERIMENTAL.

##### *Preparation of Peptides.*

The analyses of the peptides used in these experiments are summarized in Table II.

Glycyl-glycine,<sup>7</sup> glycyl-*d,l*-alanine,<sup>8</sup> glycyl-dextro-valine,<sup>9</sup> glycyl-dextro-isovaline,<sup>10</sup> glycyl-levo-leucine,<sup>11</sup> *d,l*-alanyl-glycine,<sup>12</sup> and levo-alanyl-glycine<sup>13</sup> were prepared by methods described in the literature.

<sup>6</sup> Ehrlich, F., *Biochem. Z.*, **8**, 455 (1908).

<sup>7</sup> Fischer, E., and Fourneau, E., *Ber. chem. Ges.*, **34**, 2870 (1901).

<sup>8</sup> Fischer, E., *Ber. chem. Ges.*, **37**, 2489 (1904).

<sup>9</sup> Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 138, 140 (1908).

<sup>10</sup> Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 305 (1928).

<sup>11</sup> Fischer, E., and Steingroever, J., *Ann. Chem.*, **365**, 167, 169 (1909).

<sup>12</sup> Fischer, E., *Ann. Chem.*, **340**, 128, 130 (1905).

<sup>13</sup> Fischer, E., *Ann. Chem.*, **340**, 166 (1905); also *Ber. chem. Ges.*, **40**, 507 (1907).

*d,l*-Alanyl-*d,l*-alanine<sup>14</sup> was prepared from *d,l*-bromopropionyl chloride and *d,l*-alanine by Fischer's general procedure.

*Dextro*-alanyl-*dextro*-alanine<sup>15</sup> was obtained by fractional crystallization of crude *d,l*-alanyl-*dextro*-alanine.

*Levo*-alanyl-*dextro*-alanine<sup>16</sup> was obtained from crude *d,l*-alanyl-*dextro*-alanine by concentrating the mother liquors of crystallization of *dextro*-alanyl-*dextro*-alanine.

*Glycyl*-*levo*-valine. Chloroacetyl-*levo*-valine. 23 gm. (0.2 mol) of *levo*-valine were dissolved in 100 cc. of 2.0 N sodium hydroxide (0.2

TABLE II.  
*Analysis of Peptides.*

	Sample No.	Calculated.			Found.			
		C	H	N	C	H	N	Moisture
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
Glycyl-glycine .....	2501	36 34	6 11	21 21	36 63	6 27	21 10	2 26
Glycyl- <i>d,l</i> -alanine .....	2516	41 07	6 90	19 18	41 11	6 58	19 31	1 48
<i>d,l</i> -Alanyl-glycine .....	2502	41 07	6 90	19 18	40 95	6 56	19 29	2 10
<i>Levo</i> -alanyl-glycine .....	2503	41 07	6 90	19 18	40 38	6 75	19 36	2 90
Glycyl- <i>dextro</i> -valine .....	2526	48 24	8 10	16 09	48 22	8 22	16 10	19 60
Glycyl- <i>levo</i> -valine .....	2539	48 24	8 10	16 09	48 07	8 06	16 15	1 30
Glycyl- <i>dextro</i> -isovaline .....	2528	48 24	8 10	16 09	48 30	7 80	16 00	1 01
Glycyl- <i>levo</i> -leucine .....	2530	51 03	8 57	14 89	51 24	8 52	14 88	0 84
<i>d,l</i> -Alanyl- <i>d,l</i> -alanine .....	2504	44 97	7 55	17 50	44 88	7 32	17 27	0 70
<i>Dextro</i> -alanyl- <i>dextro</i> -alanine .....	2505	44 97	7 55	17 50	45 12	7 35	17 42	1 59
<i>Levo</i> -alanyl- <i>dextro</i> -alanine .....	2506	44 97	7 55	17 50	44 92	7 39	17 15	0

mol) and treated alternately under shaking with 45 gm. (0.4 mol) of chloroacetyl chloride (b.p. 106° at 762 mm.) and 240 cc. of 2.0 N sodium hydroxide (0.48 mol) under cooling in ice, each reagent being added in equivalent proportions. The time of introduction was about

<sup>14</sup> For properties see Fischer, E., and Kautzsch, K., *Ber. chem. Ges.*, **38**, 2377 (1905).

<sup>15</sup> See Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 952, 954 (1907). Fischer, E., *Ber. chem. Ges.*, **39**, 465 (1906).

<sup>16</sup> See Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 952, 954 (1907) Fischer, E., and Raske, A., *Ber. chem. Ges.*, **39**, 3989 (1906).

1 hour. Addition of 56 cc. of 5.0 N hydrochloric acid (0.28 mol) yielded a precipitate of the chloroacetyl compound, which, after drying in a vacuum desiccator over phosphorus pentoxide, weighed 25 gm. (Yield, 65 per cent of the theory.) For analysis, the compound was recrystallized from 5 times its weight of boiling water and dried at 100° over sulfuric acid under diminished pressure. Melting point 112–113°.

0.1006 gm. substance: 0.1610 gm. CO<sub>2</sub> and 0.0574 gm. H<sub>2</sub>O.

0.1000 “ “ : 5.20 cc. 0.1 N HCl (Kjeldahl).

0.1236 “ “ : 0.0926 gm. AgCl (Carius).

C<sub>7</sub>H<sub>12</sub>O<sub>3</sub>NCl (193.61). Calculated. C 43.41, H 6.25, N 7.24, Cl 18.32.

Found. “ 43.64, “ 6.38, “ 7.28, “ 18.53.

$$[\alpha]_{\text{D}}^{25} = \frac{-1.26^\circ \times 100}{4 \times 1.94} = -16.3^\circ \text{ (in absolute alcohol);}$$

$$[\alpha]_{\text{D}}^{25} = \frac{-1.02^\circ \times 100}{4 \times 1.94} = -13.1^\circ \text{ (in absolute alcohol);}$$

0.4840 gm. in 25 cc. (0.1 M solution).

$$[\alpha]_{\text{D}}^{25} = \frac{-3.00^\circ \times 100}{2 \times 10.00} = -15.0^\circ \text{ (in absolute alcohol);}$$

1.0000 gm. in 10.0 cc. of solution.

Fischer and Scheibler<sup>17</sup> found for chloroacetyl-dextro-valine  $[\alpha]_{\text{D}}^{20} = +15.8^\circ (\pm 0.2^\circ)$ , employing a solution of 0.5051 gm. in absolute alcohol, the weight of the solution being 5.0115 gm.

Glycyl-levo-valine. 21 gm. of crude chloroacetyl-levo-valine were allowed to stand 3 days at room temperature with 210 gm. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated to dryness under reduced pressure. The residue of crude dipeptide and ammonium chloride was dissolved in 35 cc. of hot water. 500 cc. of hot absolute alcohol were added. Crystallization of the dipeptide, which set in immediately, was completed by cooling in ice. The crystals were washed with absolute alcohol. They weighed 15 gm. (Yield, 79 per cent of the theory.) For purification they were dissolved in 30 cc. of water in the presence of some norit. 300 cc. of absolute alcohol were added to the filtrate and the crystals were filtered off after standing 24 hours in ice. The product was washed with alcohol. The yield was 12.1 gm.

<sup>17</sup> Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 140 (1908).

0.0877 gm. substance: 0.1546 gm. CO<sub>2</sub> and 0.0630 gm. H<sub>2</sub>O.

0.0494 " " : 5.70 cc. 0.1 N HCl (Kjeldahl).

C<sub>7</sub>H<sub>14</sub>O<sub>3</sub>N<sub>2</sub>(174.17). Calculated. C 48.25, H 8.10, N 16.09.

Found. " 48.07, " 8.06, " 16.15.

$$[\alpha]_D^{20} = \frac{+2.03^\circ \times 100}{2 \times 5.00} = +20.3^\circ \text{ (in water);}$$

0.500 gm. in 10.0 cc. of solution.

Fischer and Scheibler<sup>18</sup> found for glycyl-dextro-valine  $[\alpha]_D^{20} = -19.7^\circ$  ( $\pm 0.2^\circ$ ), employing a solution of 0.4071 gm. in water, the weight of the solution being 4.1193 gm.

### *Preparation of Enzyme Solution.*

Clean, fresh pig intestine (10 pounds), finely ground, was suspended in glycerol (3 liters). The suspension, which was kept at 12°, was stirred at frequent intervals to insure thorough extraction of the enzyme. After 48 hours the mixture was filtered through paper in a battery of funnels, and the filtrate was stored at 0°.

For the preparation of an aqueous solution, a suitable quantity of this glycerol solution was dialyzed in collodion bags against running water for 48 hours. The cloudy solution inside the bags was filtered, and the filtrate was adjusted to pH 8.0. The same solution was used throughout the experiments.

### *Procedure in Hydrolysis Experiments.*

Samples of the peptides were weighed in volumetric flasks. To each sample were then added 0.3 equivalent of alkali and the volume of enzyme solution given in Tables III to V. The flasks were then filled to the mark with distilled water and placed in a thermostat at 37°. Samples, withdrawn at given intervals, were titrated (0.2 N NaOH) by Sørensen's formol titration method, with thymolphthalein as an indicator.

The reaction constants were calculated by means of the equation

$$kt = \log_{10} \frac{a}{a-x}$$

In the calculations  $a$  was taken as the theoretical quantity of peptide susceptible to hydrolysis. The data are given in Tables III to V.

<sup>18</sup> Fischer, E., and Scheibler, H., *Ann. Chem.*, **363**, 141 (1908).



TABLE III.

Hydrolysis of Glycyl-Glycine by Erepsin.\*

Experiment with: (1)	Control (2)	0.025 M (3)			0.050 M (4)			0.100 M (5)			0.500 M (6)			0.050 M + glycine. (7)						
		<i>t</i> hrs	<i>V</i> cc	<i>k</i> 10 <sup>3</sup>	<i>t</i> hrs	<i>V</i> cc	<i>k</i> 10 <sup>3</sup>	<i>t</i> hrs	<i>V</i> cc	<i>k</i> 10 <sup>3</sup>	<i>t</i> hrs	<i>V</i> cc	<i>k</i> 10 <sup>3</sup>	<i>t</i> hrs	<i>V</i> cc	<i>k</i> 10 <sup>3</sup>				
Glycyl-glycine, mols.	0	0	1.75	0	0	1.97	72	0	1.84	0	1.78	25	0	1.83	0	2.60	25			
Glycine, mols.	0.005	1	0 1.75	0	1	0 2.35	69	1	0 2.08	44	1	0.92	25	1	0.87	(7)	1	0 2.67	25	
Alkali, equivalents.	0.3 (glycine).	5	5	0 1.76	0	2	0 2.65	74	2	0 2.32	46	2	0.05	25	2	0.90	(6)	2	0 2.73	24
		23	0 1.74	0	4	0 3.21	74	4	0 2.72	47	4	0.31	26	4	0.93	4	3	0 2.77	21	
		48	0 1.75	0	5	0 3.43	76	5	0 2.88	47	6	0.57	27	6	0.96	4	4	2.25	2.84	22
					6	0 3.60	76	6	0 3.05	48	8	0.72	27	24	0.2	35	4	6	0 2.90	20
					7	0 3.74	77	7	0 3.22	49	24	0.3	27	48	0.2	73	4			
					8	0 3.85	76	8	0 3.36	50	47	0.4	23	73	0.4	19				
					24	0 4.47		24	0 4.13		102	0.4	21							
Average <i>k</i> · 10 <sup>3</sup> .	0			74						47		26			4					22

\* In Tables III, IV, and V, values of *k* · 10<sup>3</sup> in parentheses have not been included in calculating average values.

TABLE IV.  
*Hydrolysis of Glycyl-Peptides by Erepsin.*

Experiment with: (1)	Glycyl-D,L-alanine, 0.200 M. (2)	Glycyl-D,L-alanine, 0.100 M. (3)	Glycyl-D-tyro- sine, 0.100 M. (4)	Glycyl levo-valine, <sup>a</sup> 0.100 M. (5)	Glycyl-D-tyro- sine, 0.100 M. (6)	Glycyl-levo-leucine 0.100 M. (7)																																																																																																																																																																																																
Peptide, mols.	0.005	0.005	0.005	0.005	0.005	0.005																																																																																																																																																																																																
Alkali, equivalents.	0.3	0.3	0.3	0.3	0.3	0.3																																																																																																																																																																																																
Erepsin solution, cc.	2.5	5.0	5.0	5.0	5.0	5.0																																																																																																																																																																																																
Total volume, cc.	25.0	50.0	50.0	50.0	50.0	50.0																																																																																																																																																																																																
Samples, cc.	2.5	5.0	5.0	5.0	5.0	5.0																																																																																																																																																																																																
	<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.76</td><td></td></tr><tr><td>1</td><td>1.85 (33)</td><td></td></tr><tr><td>2</td><td>1.98 42</td><td></td></tr><tr><td>3</td><td>2.07 41</td><td></td></tr><tr><td>4</td><td>2.13 38</td><td></td></tr><tr><td>5</td><td>2.22 40</td><td></td></tr><tr><td>6</td><td>2.28 39</td><td></td></tr><tr><td>8</td><td>2.41 40</td><td></td></tr><tr><td>23</td><td>2.97</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.76		1	1.85 (33)		2	1.98 42		3	2.07 41		4	2.13 38		5	2.22 40		6	2.28 39		8	2.41 40		23	2.97		<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.74</td><td></td></tr><tr><td>0.5</td><td>1.88 (104)</td><td></td></tr><tr><td>1</td><td>1.98 (93)</td><td></td></tr><tr><td>1.5</td><td>2.06</td><td></td></tr><tr><td>2</td><td>2.15</td><td></td></tr><tr><td>2.5</td><td>2.23</td><td></td></tr><tr><td>3</td><td>2.25</td><td></td></tr><tr><td>4</td><td>2.35</td><td></td></tr><tr><td>6</td><td>2.76 (123)</td><td></td></tr><tr><td>25</td><td>3.24</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.74		0.5	1.88 (104)		1	1.98 (93)		1.5	2.06		2	2.15		2.5	2.23		3	2.25		4	2.35		6	2.76 (123)		25	3.24		<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.74</td><td></td></tr><tr><td>0.5</td><td>1.84 (35)</td><td></td></tr><tr><td>1</td><td>1.89 (27)</td><td></td></tr><tr><td>2</td><td>2.00 24</td><td></td></tr><tr><td>3</td><td>2.08 21</td><td></td></tr><tr><td>4</td><td>2.17 20</td><td></td></tr><tr><td>5</td><td>2.36 22</td><td></td></tr><tr><td>7</td><td>2.49 22</td><td></td></tr><tr><td>26</td><td>3.70 (26)</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.74		0.5	1.84 (35)		1	1.89 (27)		2	2.00 24		3	2.08 21		4	2.17 20		5	2.36 22		7	2.49 22		26	3.70 (26)		<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.76</td><td></td></tr><tr><td>1</td><td>1.75</td><td></td></tr><tr><td>5</td><td>1.79</td><td></td></tr><tr><td>7.5</td><td>1.79</td><td></td></tr><tr><td>24</td><td>1.81</td><td></td></tr><tr><td>55</td><td>1.87</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.76		1	1.75		5	1.79		7.5	1.79		24	1.81		55	1.87		<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.75</td><td></td></tr><tr><td>0.5</td><td>1.75</td><td></td></tr><tr><td>1</td><td>1.74</td><td></td></tr><tr><td>2</td><td>1.74</td><td></td></tr><tr><td>3</td><td>1.76</td><td></td></tr><tr><td>4</td><td>1.77</td><td></td></tr><tr><td>5</td><td>1.77</td><td></td></tr><tr><td>7</td><td>1.80</td><td></td></tr><tr><td>26</td><td>1.89</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.75		0.5	1.75		1	1.74		2	1.74		3	1.76		4	1.77		5	1.77		7	1.80		26	1.89		<table><tr><td><i>t</i></td><td><i>V</i></td><td><i>k</i> 10<sup>4</sup></td></tr><tr><td><i>hrs.</i></td><td><i>cc.</i></td><td></td></tr><tr><td>0</td><td>1.72</td><td></td></tr><tr><td>0.5</td><td>1.82 35</td><td></td></tr><tr><td>1</td><td>1.89 31</td><td></td></tr><tr><td>2</td><td>2.05 31</td><td></td></tr><tr><td>3</td><td>2.21 32</td><td></td></tr><tr><td>4</td><td>2.35 32</td><td></td></tr><tr><td>5</td><td>2.58 33</td><td></td></tr><tr><td>7</td><td>2.78 34</td><td></td></tr><tr><td>26</td><td>3.94 36</td><td></td></tr></table>	<i>t</i>	<i>V</i>	<i>k</i> 10 <sup>4</sup>	<i>hrs.</i>	<i>cc.</i>		0	1.72		0.5	1.82 35		1	1.89 31		2	2.05 31		3	2.21 32		4	2.35 32		5	2.58 33		7	2.78 34		26	3.94 36	
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TABLE V.  
*Hydrolysis of Alanyl-Peptides by Erepsin.*

Experiment with- (1)	<i>d, l</i> alanyl-glycine, 0.200 M (2)				<i>d, l</i> alanyl-glycine, <sup>†</sup> 0.100 M (3)				Levo-alanyl- glycine, <sup>†</sup> 0.100 M (4)				<i>d, l</i> alanyl- <i>d, l</i> alanine, 0.100 M (5)				Dextro alanyl-dextro- alanine, 0.100 M (6)				Levo-alanyl- dextro-alanine, <sup>†</sup> 0.100 M (7)			
	<i>t</i>		<i>k</i> 10 <sup>3</sup>		<i>t</i>		<i>k</i> 10 <sup>3</sup>		<i>t</i>		<i>k</i> 10 <sup>3</sup>		<i>t</i>		<i>k</i> 10 <sup>3</sup>		<i>t</i>		<i>k</i> 10 <sup>3</sup>		<i>t</i>		<i>k</i> 10 <sup>3</sup>	
	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>
Peptide, mols.	0	1.83	56		0	1.76	15 <sub>1</sub>		0	1.78	0		0	1.78	0		0	1.73	0		0	1.75	0	
Alkali, equivalents.	1	0.198	56		0.5	1.96	15 <sub>1</sub>		1	0.187	0		1	0.181	0		0.5	2.05	(11 <sub>6</sub> )		1	0.178	0	
Erepsin solution, cc.	2	0.214	62		1	0.211	14 <sub>6</sub>		2	0.194	0		2	0.182	(13)		1	0.242	14 <sub>6</sub>		2	0.179	0	
Total volume, cc.	3	0.225	59		1.5	2.24	14 <sub>6</sub>		5	0.196	0		5	0.186	(11)		1.5	2.74	15 <sub>6</sub>		5	0.178	0	
Samples, cc.	4	0.236	60		2	2.52	41	16 <sub>1</sub>	25	0.202			6	0.186	9		2	0.303	16 <sub>6</sub>		25	0.192		
	5	0.242	65		3	0.263	16 <sub>6</sub>		53	0.210			24	0.197	6		2.5	3.27	16 <sub>6</sub>		53	0.207		
	6	0.253	59		4	0.289	(25 <sub>1</sub> )						25	0.204	8		3	3.64	(19 <sub>6</sub> )					
	7	0.262	62		6	0.292	(19 <sub>6</sub> )						47	0.218	8		6	0.409	(20 <sub>7</sub> )					
	23	0.302			25	0.302							53	0.224	9		25	0.416						
					47	0.323							73	0.241	(15)									
					73	0.347							102	0.257										
					102	0.355																		
Average <i>k</i> · 10 <sup>3</sup> .		60					15 <sub>1</sub>				0				8								15 <sub>6</sub>	0

\* The inferior numbers are used to indicate figures which are not significant.

Control experiments with 0.100 M glycyl-glycine were run for each series of hydrolyses; the hydrolysis constants of these controls were identical within the limits of error.

*Hydrolysis of d,l-Alanyl-Glycine.*

A solution of 0.005 mol of peptide, 0.3 equivalent of alkali, and 5.00 cc. of enzyme solution was made up to a volume of 25.0 cc. A sample of 2.50 cc. required (formol titration) 1.76 cc. of 0.2 N alkali. A sample of 5.00 cc. was neutralized with the theoretical quantity of hydrochloric acid, filtered, and diluted to 10.0 cc. The rotation in a 1 dm. tube was  $\alpha_D^{25} = 0^\circ$ .

The original solution was then allowed to stand 20 hours at  $37^\circ$ . A sample of 2.50 cc. required (formol titration) 2.98 cc. of 0.2 N alkali. A sample of 5.00 cc. was neutralized with the theoretical quantity of hydrochloric acid, filtered, and diluted to 10.0 cc. The rotation in a 1 dm. tube was  $\alpha_D^{25} = -0.38^\circ$ .

The calculated rotation of a mixture of levo-alanyl-glycine and dextro-alanine equivalent to the above solution is  $\alpha_D^{25} = -0.36^\circ$ .

## BLOOD SUGAR DETERMINATION AND SEPARATION OF SUGARS WITH LIVE YEAST.

### A CORRECTION.

BY ALBERT L. RAYMOND AND J. G. BLANCO.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 22, 1928.)

In connection with the paper bearing the above title,<sup>1</sup> Dr. Stanley R. Benedict has called our attention to his discussion, which we had overlooked, of Somogyi's conclusion that some sugars may be removed from dilute solution through adsorption by living yeast cells.<sup>2</sup> In view of the evidence which Benedict has presented, favoring the view that the process of removal of sugars from dilute solution by live yeast is solely one of fermentation, it seems desirable to substitute the term "disappearance" for "adsorption" as used in our paper.

Our interest lay in the rapid and selective nature of the process and we made no attempt to investigate its mechanism or to distinguish between adsorption, diffusion, fermentation, *etc.*

Attention should be called to the importance of avoiding too low a temperature in employing the method which we described, 18-20° being probably the safe lower limit. Temperatures much below this, Benedict has shown, considerably reduce the rate of disappearance of glucose.

<sup>1</sup> Raymond, A. L., and Blanco, J. G., *J. Biol. Chem.*, 1928, lxxix, 649.

<sup>2</sup> Benedict, S. R., *J. Biol. Chem.*, 1928, lxxvi, 464.



## THE VISCOSITY OF BLOOD SERUM, AS A FUNCTION OF TEMPERATURE.

By P. LECOMTE DU NOÛY.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York,  
and the Pasteur Institute, Paris, France.)

(Accepted for publication, June 16, 1928.)

The viscosity of colloidal solutions is not easily determined by the methods which involve the use of a glass capillary. The technical difficulties encountered are important, and when they are successfully surmounted they still require a relatively large amount of liquid. Moreover, they do not permit the continuous observation of the variations which may take place in a solution as a consequence of a reaction, or under the influence of temperature, or of time. These were the main reasons which led us to establish a viscometer which would escape the above limitations. Such an instrument was described a few years ago.<sup>1</sup> Suffice it to say that it is based on the principle of two coaxial cylinders; the outside cylinder or cup, which contains about 1 cc. of the solution to be studied, is rotating at constant speed, and the inside cylinder, or bob, is suspended by means of a galvanometer wire. A mirror, supported by the suspension, a lamp and scale for the readings and a good thermostatic arrangement, are provided.

The purpose of the present paper is to report the results of roughly 167 series of experiments, representing about 17,000 readings of viscosity, performed with normal blood serum (rabbit, dog, horse), and to discuss the interpretation of some of the data obtained, with especial reference to the hydration of the serum proteins.

### EXPERIMENTAL.

In order to save the reader's time, and to make him visualize a series of experiments at a glance, only charts will be published. The experiments chosen for publication are representative of the others, as no radical departure from the mean has been observed so far.

<sup>1</sup> du Noüy, P. L., *J. Gen. Physiol.*, 1923, v, 429.

The experiments were carried on in the following way: the serum was poured into the cup (1 cc.) and the bob lowered into the serum. The zero being checked, the motor was started, and a first reading made at the starting temperature (between 20° and 25°C.). After checking the zero again, and repeating this measurement at least 3 times, with an interval of 5 minutes between each reading, the heat was put on. (A current of 1 ampere through a resistance immersed in the oil surrounding the cup brought the temperature up from 20° to 70° in about 45 minutes.) Then the readings were taken, and recorded simultaneously with the temperature. A telescope placed alongside of the scale makes this possible. As a rule, unless some critical point was neared or some unforeseen phenomenon occurred, readings were taken every 2°. The current was left on until the temperature of 70°C. was reached, in the first series of experiments (Figs. 1 and 2); in all other experiments reported in this paper the heat was stopped after a certain temperature had been reached (50°, 55°, 56° to 60°) and the oil allowed to cool by itself. As a rule, in order that the cooling from, say, 55° to 20°, should take about the same time as the heating from 20° to 55°, cold water was made to circulate in the double wall of the oil bath, and the rate controlled accordingly.

The first set of experiments is shown in Fig. 1. The ordinates express the readings on the scale, and therefore are arbitrary figures, but proportional to the absolute viscosities. In order to give an idea of the order of magnitude, the curve expressing the viscosity of water as a function of temperature is drawn below.

A simple glance at this chart immediately reveals the presence of a critical point, corresponding to an absolute minimum of viscosity, at a temperature near 56°. Had we not known beforehand that this was a critical temperature for the serum, from a biological standpoint, we could not have failed to notice it from these curves.

Fig. 2 illustrates the same phenomenon. The turning point may vary, from 56° to 58°. But in the great majority of cases, the viscosity reaches its minimum value between 56° and 57°, stays constant up to 58°, sometimes up to 59°, and begins to increase more rapidly than it decreased before. From 62° to 65° the increase becomes very rapid, and it sometimes happens that the spot has left the scale before 70° is reached. This is usually the case with horse serum (which is normally more viscous than rabbit serum). It happens rarely in the case of rabbit serum. Fig. 2 shows that it was necessary to keep the temperature at 70° for 2 minutes in order to send the spot off the scale. It is to be noted that when fresh serum is used, important fluctuations



are frequently observed around  $45^{\circ}$  to  $55^{\circ}$ . These were *never* observed when the serum had been submitted to a heat of  $55^{\circ}$ , even for 5

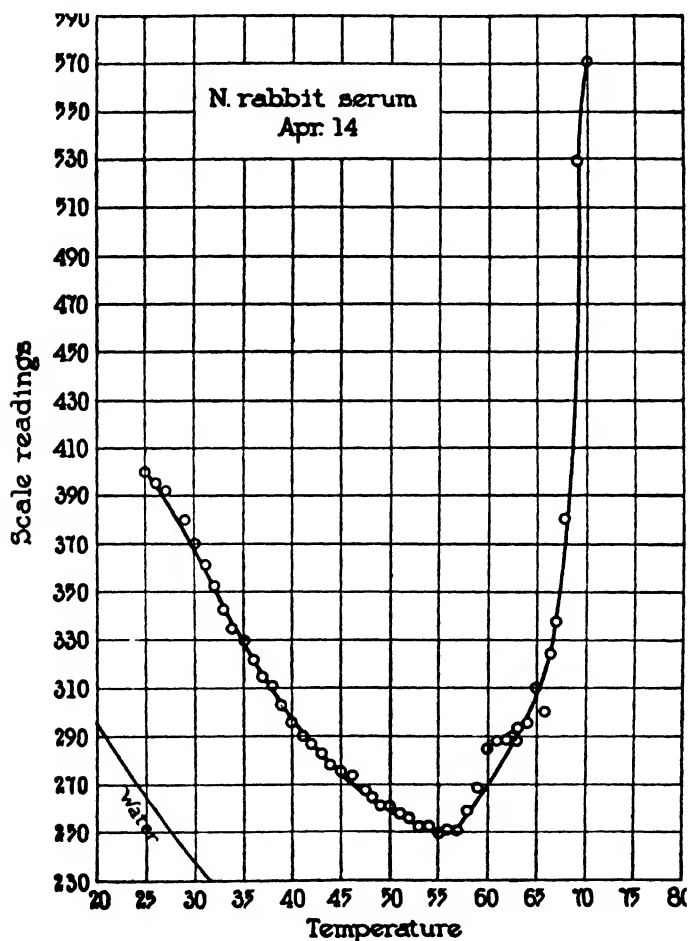


FIG. 1.

minutes, or when it was old. It appeared to us that it might be interesting to follow the phenomenon more closely, and to try and de-

termine whether the temperature played a specific part in the changes of viscosity, or whether the time of heating was the capital factor. In other words whether heating for 15 minutes at 55° would produce the same increase in viscosity as 5 minutes at 60°, for example. The first experiment was made with a serum heated at 55° for 10 minutes in a sealed tube. It is obvious from Fig. 2 that there is no fundamental change in the curve: the two control curves (white and black circles) almost coincide with that of the heated serum.

Another similar experiment is shown in Fig. 3. However instead of carrying the heating on up to the time when the spot goes off the scale, it was stopped, and the liquid was allowed to cool, according to the technique previously described. The white circles express the values of the heated serum 1 hour at 50°. The black circles are the values taken by the heated serum, on cooling, after having been kept at 57° for 5 more minutes. In general, no difference can be detected between heated and unheated serum when the serum is not heated above 55°. Up to 50° the curve expressing the viscosity of serum as a function of temperature is parallel to that of pure water, and its proteins play no part at all, or rather act only by their bulk, to displace the curve as a whole. From 50° on, a slight departure is observed; it goes on increasing until the minimum value is attained around 56°. Heating for 1 hour at 50° fails to alter permanently the viscosity of serum. The phenomenon is entirely reversible, as in the preceding case (Fig. 3).

Heating for 15 minutes at 55° acts in the same way. But as the heat was brought up to 56° in one series of measurements, while it was stopped at 55° in the other (Fig. 4) a small but marked difference could be detected between the two cooling curves. Taking evaporation into consideration, the slight increase in viscosity of the serum heated up to 55° can be accounted for, but the difference between this sample and the sample brought up to 56° is due to something else. Here the phenomenon is no longer reversible; the relative viscosity reaches 1.70. The same experiment was repeated with the same serum, heated for 15 minutes at 56° in a sealed tube. The mean viscosity was 1.70. The increase is small, but constant with this serum. It was not always observed with other sera. We can therefore state that, from our experiments, it appears that 56° is the lowest

temperature at which an irreversible phenomenon affecting its viscosity occurs in rabbit serum, in 15 minutes. However, an exception to this rule was found once. But if the heat is kept for 30 minutes at  $56^{\circ}$  (Fig. 5) the mean value of  $\eta$  climbs up to 1.77 and higher still on cooling. On the other hand in certain cases, 5 minutes at  $56^{\circ}$  (dog serum) may bring forth no change at all.

Another serum, the viscosity of which was normally high, heated up to  $58^{\circ}$  and cooled immediately after that temperature was reached, showed no modification. Serum 3 ( $\eta = 1.63$ ) heated at  $58^{\circ}$  for 15 minutes, gave similar curves with  $\eta = 1.80$ . Half an hour at  $58^{\circ}$  brings the value of  $\eta$  up to 1.95.

Another serum heated for 1 hour, and 2 hours, at  $58^{\circ}$  (Fig. 6) reaches a viscosity of 1.85 (mean value) with a maximum of 1.90. 1 hour's heating of this serum at  $60^{\circ}$  fails to affect it more than 1 hour at  $58^{\circ}$ .

Fig. 7 is self-explanatory. The serum was heated in the cup, for 5 minutes, in all cases. As the temperature goes up, the different samples show a similar behavior. But on cooling, the differences between the different curves, according to the temperature reached, go on increasing. However, up to  $62^{\circ}$  no sign of structure in the liquid was found. The zero checked perfectly, and what was measured was true viscosity.

Fig. 8 summarizes for one animal a whole series of observations, showing which combinations of temperature and of time determined the same increase in viscosity. The question then arises as to what the increase in viscosity is due to, and how it can be interpreted.

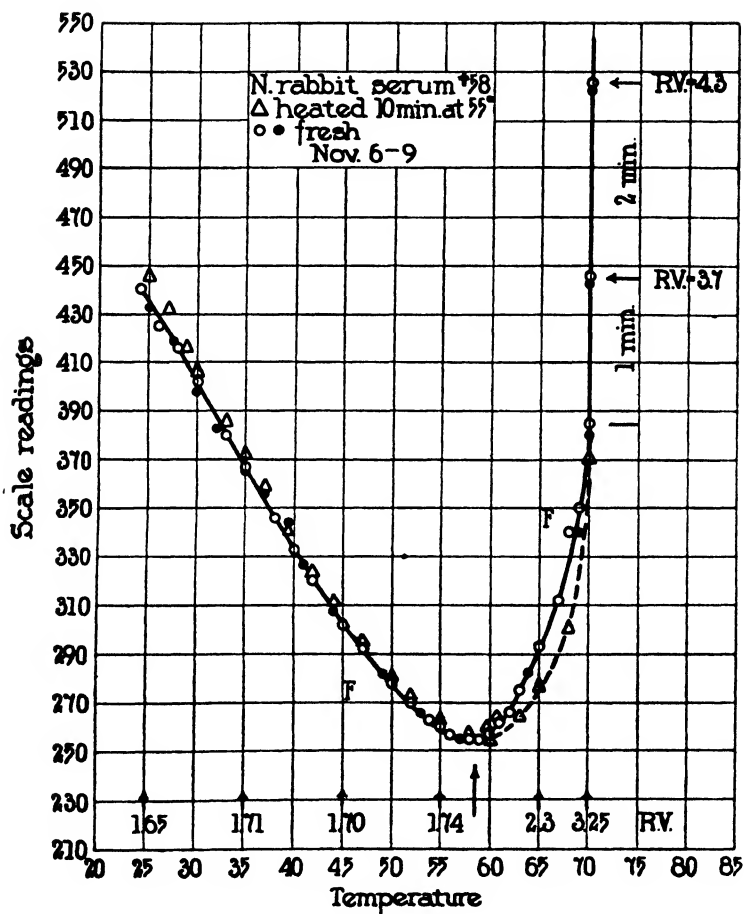


FIG. 2.

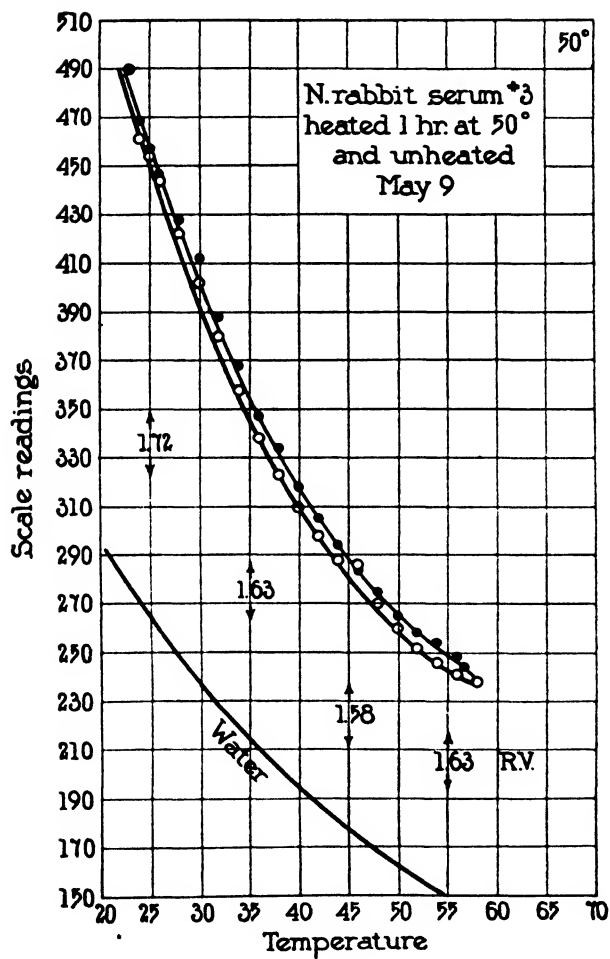


FIG. 3.

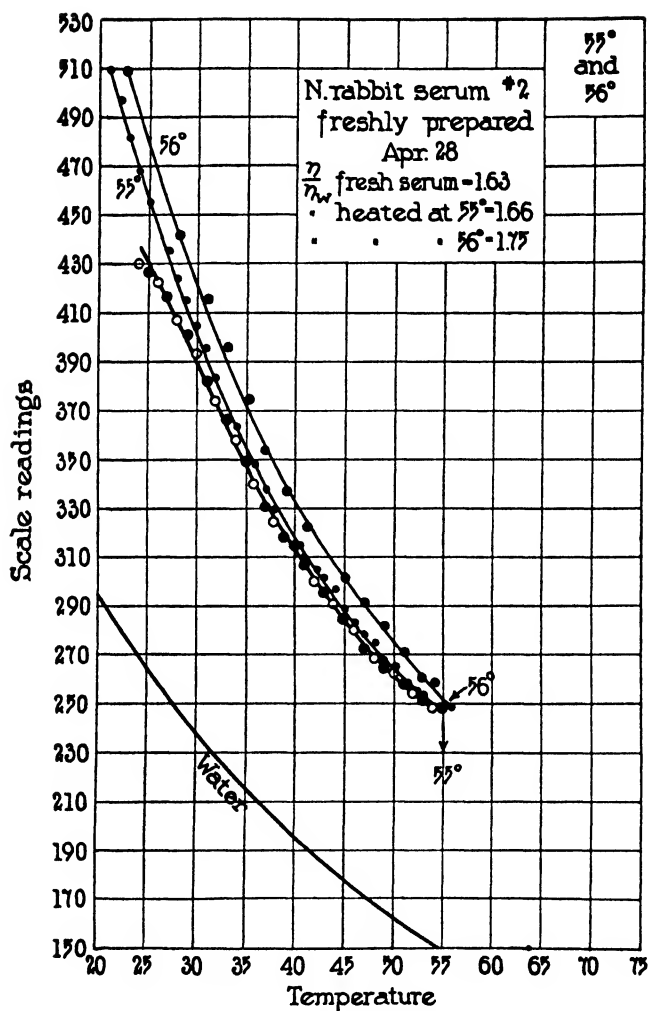


FIG. 4.

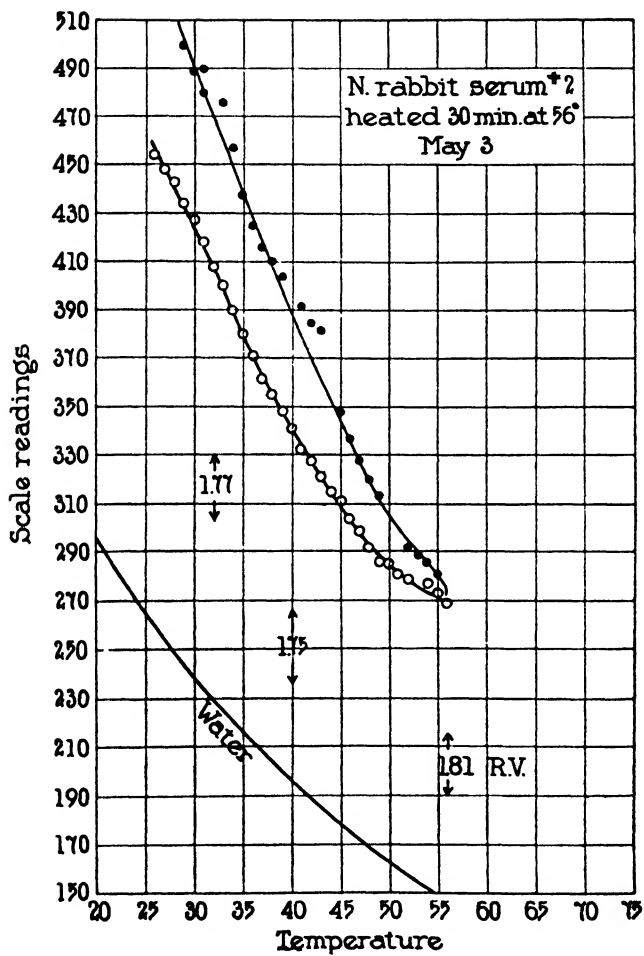


FIG 5.

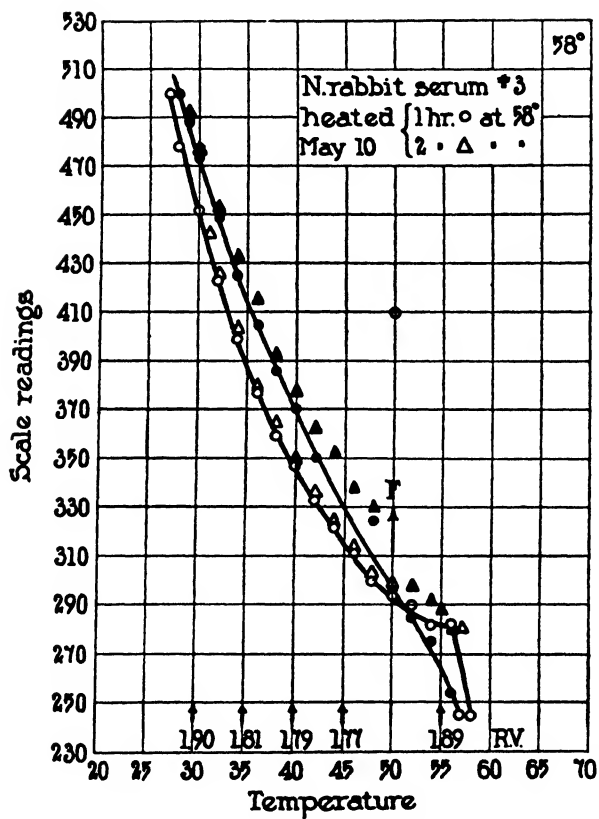


FIG. 6.



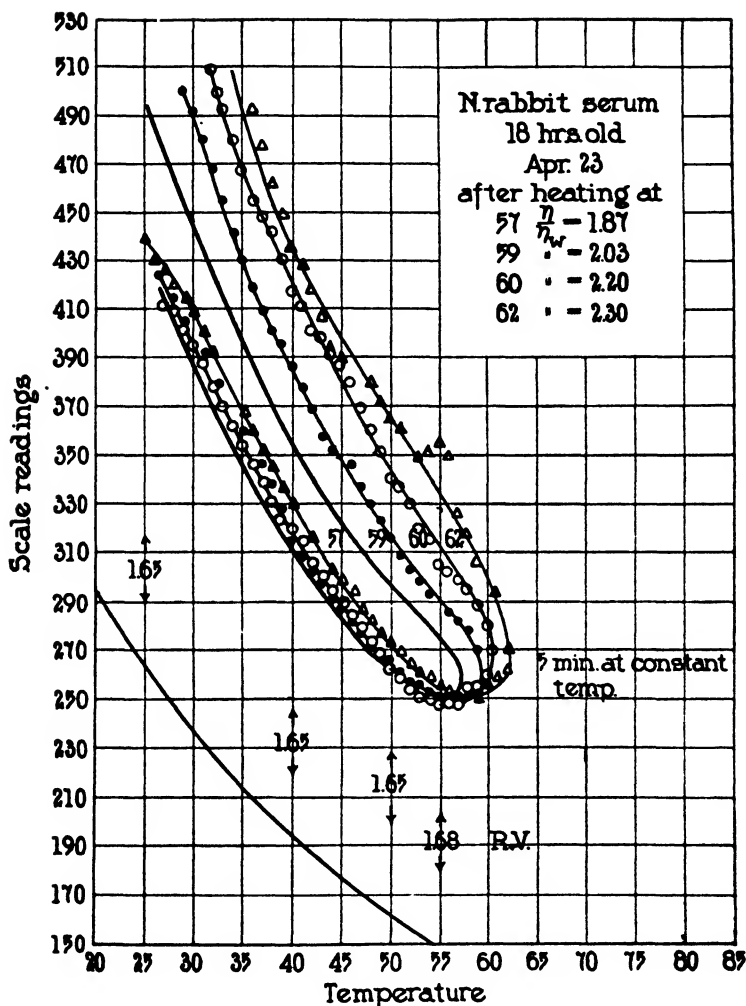


FIG. 7.

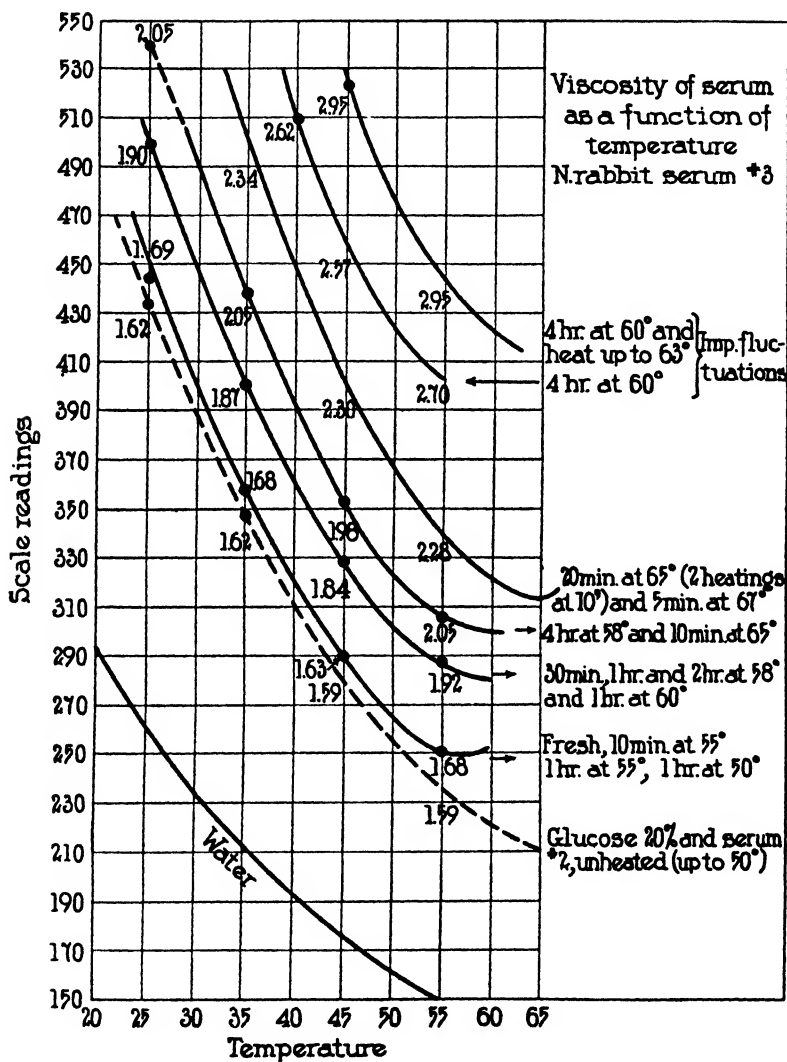


FIG. 8.

## DISCUSSION.

In our experiments the concentration remains constant, yet the viscosity increases. On the other hand, Einstein<sup>2</sup> states that the degree of dispersion, *i.e.*, the size of the molecules or particles is immaterial, and that viscosity can be expressed as a linear function of the volume fraction of the dispersed substance, according to the formula

$$\eta = 1 + 2,5 \varphi \quad (1)$$

which is an approximation of the expression

$$\eta = \frac{1 + 0.5 \varphi}{(1 - \varphi)^2} \quad (2)$$

where  $\eta$  stands for the relative viscosity of the suspension, and  $\varphi$  for the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution. These equations, however, fail to express the experimental facts. The reason for this lack of agreement is not clear. But Kunitz has recently proposed an empirical formula which applies remarkably well to a number of widely different cases, including lyophilic and lyophobic sols, and within large limits of concentration. This formula is<sup>3</sup>

$$\eta = \frac{1 + 0.5 \varphi}{(1 - \varphi)^4} \quad (3)$$

The problem we are facing can be stated as follows: Assuming that Einstein's view concerning the part played by  $\varphi$  as defined above is correct, and that the degree of dispersion is immaterial, we can use formula (1), and compute the values of  $\varphi$ , and consequently determine  $\frac{\varphi}{C}$  which expresses the specific volume of the solute (proteins). These values divided by the specific volume of the dry proteins should give the amount of hydration of the proteins in solutions, at temperatures up to 55°, and its increase as a function of temperature. But we know that this formula does not apply to lyophilic sols at high concentration, consequently, we cannot rely on the figures at all.

<sup>2</sup> Einstein, A., *Ann. Physik.*, 1906, xix, 289; 1911, xxxiv, 591.

<sup>3</sup> Kunitz, M., *J. Gen. Physiol.*, 1926, ix, 715.

On the other hand we can apply Kunitz's formula (Table I) which we know fits the experimental facts very satisfactorily but then the main assumption of Einstein concerning the rôle of the degree of dispersion, which is a consequence of his mathematical derivations, may not hold any longer. Therefore, it does not seem possible, at present, to decide whether the figures computed in this way express quantitatively the increase due to hydration alone or whether some other

TABLE I.

*Viscosity of Rabbit Serum 3, Heated at Different Temperatures, and Values of  $\varphi$  and  $\frac{\varphi}{C}$  Computed from Kunitz's formula (see Figs. 7 and 8).*

$C$  = mean concentration of proteins in the serum = 6.5 per cent.

1	2	3	4	5	6	7
Relative viscosity $\frac{\eta}{\eta_0}$	$\varphi$	Specific volume $\frac{v}{c}$	Specific volume of dry proteins	Ratio Column 3 Column 4	Increase per cent of specific volume (Hydration ?)	Increase in hydration due to heating
	<i>per cent</i>				<i>per cent</i>	<i>per cent</i>
1.65	10.7	1.645	0.785	2.09	109	Unheated
1.69	11.2	1.720	"	2.19	119	
1.88	13.3	2.030	"	2.58	158	
2.03	14.8	2.275	"	2.90	190	
2.30	17.2	2.650	"	3.37	237	
2.62	19.8	3.050	"	3.88	288	
2.95	21.8	3.350	"	4.27	327	208

phenomenon is also responsible for it to a certain extent. However, column 6 has been tentatively designed as "hydration."<sup>4</sup>

<sup>4</sup> The influence of the charge of colloidal particles on the viscosity of the sol has been taken in consideration, especially by von Smoluchowski (*Kolloid-Z.*, 1916, xviii, 194.) who enlarged Einstein's formula which became:

$$\eta = \eta_M \left\{ 1 + 2.5 \varphi \left[ 1 + \frac{1}{\lambda \eta_M r^2} \left( \frac{D \zeta}{2 \pi} \right) \right] \right\}$$

however our results are not in accord with this formula, which should lead to a lower viscosity as the size of the particles increases. We observe the contrary, and there seems to be no doubt that after heating the size of the particles increases, since coagulation is the limit of the phenomenon. Perusal of Freundlich's excellent discussions of the subject in general (*Colloid and capillary chemistry*, New York, pp. 367 and following; 539, etc.) will repay the reader.

All that can be said is that it expresses the increase per cent of the specific volume of the serum proteins. It is interesting to note that the figures expressing "hydration" obtained from Einstein's formula (3) are larger (3.71 times) than those obtained from Kunitz's formula (1), and that this ratio remains constant up to a viscosity of 2.03. From this figure up the discrepancy begins and increases rapidly with increasing viscosity.

Fig. 8 suggests another observation: the dotted curve (viscosity 1.62) was obtained with a sugar solution (glucose 20 per cent). The concentration is 3 times that of the serum. Hence, serum may be said to behave exactly like a true solution, as a function of temperature, up to nearly 55°. It is surprising that such a concentrated solution of proteins should have such a low viscosity. When, by diluting the serum, its specific volume is made equal to that of the sugar solution, its viscosity is much inferior to that of the latter.



## THE INJECTION OF SULFATES INTO VALONIA.

By L. R. BLINKS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, May 24, 1928.)

Cells of *Valonia macrophysa*, while ordinarily free from sulfate in the vacuolar sap,<sup>1</sup> occasionally are found in apparently normal state yet giving a test for this ion. In order to determine its effect directly, the following experiments were performed in Bermuda in 1924. The number of cells was small (30) but the results sufficiently definite to justify certain conclusions. The method furthermore is of interest in view of its later employment for the injection of toxic substances.<sup>2</sup>

Two successive operations were employed. A small amount of sap (about 1/8 of the cell volume) was withdrawn through a fine capillary. This was removed and a second capillary inserted, containing the solution for injection. The contents were forced in under air pressure (taking care that no bubbles entered) until the cell was turgid. The capillary was removed while the pressure was still being applied.

Mortality was high due to the two punctures and the consequent temporary softness of the cell, but a small number of cells survived in both of two groups. In group A a solution of 0.6 M KCl was injected. Of these 2 cells survived and lived 2 weeks, when the experiment was discontinued. In group B a solution of 0.4 M K<sub>2</sub>SO<sub>4</sub> was injected, and 3 cells survived. At the end of 2 weeks the sap was removed from all the cells and tested for sulfate ion. Group A gave no test, while group B gave a very strong one (approximately as much as the surrounding sea water). The cells were turgid and seemed normal in every respect. Hence, as far as we can judge from these experi-

<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, v, 225.

<sup>2</sup> Rapkine and Wurmser have also reported the injection of dyes into the vacuole of *Spirogyra*, using the method of Chambers as modified by the Needhams (Rapkine, L., and Wurmser, R., *Proc. Roy. Soc. London, Series B*, 1927, cii, 128).

ments, the protoplasm can tolerate a considerable amount of sulfate on the vacuolar as well as on the outer surface.

It is also evident that there is no mechanism for rapidly disposing of sulfate after it has entered. If, therefore, the cells have in the past suffered mechanical or other injury which temporarily increased the permeability of sulfates, recovery would still leave a certain amount of these in the vacuole, without subsequent injury to the cell.

#### SUMMARY.

Potassium chloride and sulfate were injected into the vacuole of *Valonia*. The surviving cells tolerated the presence of these solutions. Sulfate, although ordinarily absent from the sap, is not rapidly eliminated when introduced. Hence the sulfate occasionally found in cells of normal appearance may have entered due to temporary injury followed by recovery.



## INTERNAL VERSUS EXTERNAL TOXICITY IN VALONIA.

BY A. G. JACQUES AND W. J. V. OSTERHOUT.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 5, 1928.)

Our bioelectric measurements indicate that in *Nitella* and *Valonia* the protoplasm consists of layers,<sup>1</sup> of which the inner is more sensitive to the action of chloroform than the outer (when both are in contact with the same electrolytes at the same concentration).<sup>2</sup>

It is a matter of considerable interest to determine the relative sensitivity of these layers by applying a toxic substance to each one separately. The experiments here described were made for this purpose. The cells were divided into two groups. Those of group *A* were allowed to stand in sea water containing a small amount of the toxic agent so that only the outer layer (*X*) was directly exposed. In group *B* sap containing the toxic agent was injected into the cells.<sup>3</sup> Here only the inner layer (*Y*) was directly exposed. It seems probable that both *X* and *Y* are relatively impermeable to the toxic agent here employed ( $\text{MnCl}_2$ ), at least as long as the protoplasm is not severely injured, and if this is the case we may expect death to occur more rapidly when the poison is applied to the more sensitive layer.<sup>4</sup>

The experiments were carried out in Bermuda on cells of *Valonia macrophysa* (containing from 0.3 to 1.2 cc. of sap) at about 20-25°C.

It was necessary to find a toxic agent which could be measured with a fair degree of accuracy even in very small amounts. Such organic reagents as chloroform or formaldehyde were ruled out because of analytical difficulties. After a number of trials in which  $\text{Ni}^{++}$ ,  $\text{Cd}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Fe}^{+++}$ ,  $\text{Cr}^{++}$ , and  $\text{Mn}^{++}$  were investigated our choice fell on the last. From the analytical point of view Mn is excellent, since

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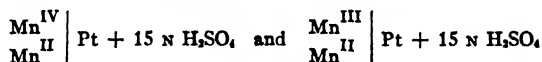
<sup>1</sup> It is assumed that there is an inner layer *Y* and an outer layer *X* both of which are probably non-aqueous: between them there is an aqueous layer *W*.

<sup>2</sup> Cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.

<sup>3</sup> Cf. Blinks, L. R., *J. Gen. Physiol.*, 1928-29, xii, 207.

<sup>4</sup> But if the protoplasm were freely permeable to the toxic agent it would make little difference whether it is injected into the cell or applied from the outside.

it can be determined to 0.01 mg. with good accuracy. It has, however, several valences (2, 3, 4, 6, and 7). In our experiments it was employed in the form of the bivalent salt,  $\text{MnCl}_2$ . This may be considered as the salt of a fairly strong base and a strong acid since it undergoes no appreciable hydrolysis. A possible objection is the uncertainty in regard to oxidation. In the sea water the chance of oxidation must be very small. On the other hand we know very little about the oxidation conditions within the vacuole of the cell. We do know, however, that the normal oxidation potentials of the electrodes



are strongly positive: according to Grube and Huberich<sup>5</sup> over 1.5 plus referred to the hydrogen electrode taken as zero. This indicates such a strong tendency of the higher valence compound to be reduced that it does not seem likely that the cell can oxidize  $\text{MnCl}_2$ .

It was hoped at the start that we might be able to determine the maximum concentration of  $\text{Mn}^{++}$  within the vacuole which the cell would tolerate indefinitely.<sup>6</sup> This was not realized. A considerable number of cells were found which fulfilled the conditions required, but on analysis they were found to contain so little Mn that the results were very inaccurate. In 17 out of 20 cases where the cells lived for a long time not more than a trace was found. For a similar reason we were unable to determine very accurately the maximum concentration of  $\text{MnCl}_2$  in the sea water that the cell would tolerate indefinitely. The plan was therefore adopted of injecting the cells in group A with sap containing  $\text{MnCl}_2$ , and allowing them to stand in sea water until dead.<sup>7</sup> Analyses were then made of the cell contents. In this way data were obtained giving the length of life of cells containing 0.004 to 0.185 per cent of  $\text{MnCl}_2$  in their sap. In group B the cells (stabbed with a capillary but not injected with  $\text{MnCl}_2$ ) were simply placed in sea water containing  $\text{MnCl}_2$  and allowed to remain until dead.

### *Technique.*

In a previous paper<sup>8</sup> we have stated that occasionally cells which had been impaled on a capillary and then shaken from the capillary into sea water, healed

<sup>5</sup> Grube, G., and Huberich, K., *Z. Elektrochem.*, 1923, xxix, 8.

<sup>6</sup> "Indefinitely" is here used in a limited sense. We know that cells will live for periods of months in sea water in the laboratory, without showing any change in the protoplasm except a slight fading of the color. In this work "indefinitely" simply indicates a period of a month or more during which there is no more change in the appearance than would be displayed by a cell not exposed to Mn.

<sup>7</sup> The sense in which this word is employed is explained on page 215.

<sup>8</sup> Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, xi, 202.

up, becoming as turgid as unpunctured cells and continuing to live. This observation formed the basis of our present work. It was found that the proportion of punctured cells which could be made to heal could be very greatly increased by careful handling. Glass capillaries were used, and these were attached to a Luer hypodermic syringe of 2 cc. capacity. The capillaries were considerably finer than those used by us in making the measurements of potential difference. The glass at the tip was extremely thin, and the point was broken off at an angle to facilitate the piercing of the relatively tough cellulose wall. During injection the syringe was filled with sap containing a definite concentration of  $MnCl_2$ . The cell was then grasped firmly between the thumb and forefinger and quickly impaled on the capillary and at the same time a slight pressure was applied to the plunger of the syringe. As soon as the cell was in place on the capillary it was

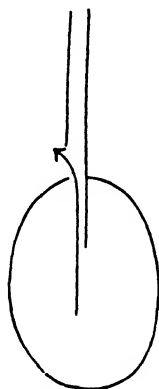


FIG. 1.

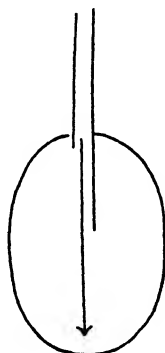


FIG. 2.

FIGS. 1 and 2. Methods of inserting a hollow steel needle into the cell.

relinquished by the fingers, and then a considerable pressure was applied to the piston. At this stage, those cells which showed any sign of leakage around the capillary were rejected. This was done because any severe leak indicated that an undue displacement of the natural sap by artificial sap plus  $MnCl_2$  had probably occurred, and also because when a cell leaked on puncturing there was usually some scouring and tearing of the protoplasm at the site of the leak. This was probably due to the sudden rush of sap out through the minute annular space. Cells so damaged usually failed to heal. The passage of sap +  $MnCl_2$  into the cell could be observed owing to the difference of refractive index of the solutions. It was thus possible to control in a very rough way the amount injected. It is obvious that at the moment of injection the pressure in the vacuole must have been above normal. Nevertheless in only a very few cases (excepting those cells which leaked at the puncture) was there any evidence of rupture of the cell wall.

Further it is of interest to note that those cells which showed any sign of such rupture were invariably large, containing 0.75 cc. or more. The actual transfer of the solution from the syringe to the cell took only a few seconds. While the pressure was still maintained the cell was brushed quickly from the capillary into a large volume of sea water.

This method of injection yielded the greatest number of cells which healed. Deflation of the cell was completely avoided. A second method which was tried and abandoned involved the use of a very fine hollow steel needle. This was ground off at the point at a steep angle. The cell was brought to the needle and forced up on it rather slowly. As Fig. 1 shows there was at the start a passage through which sap could escape as indicated by the arrow. This passage was closed as soon as the impalement was complete (Fig. 2). By applying a slight pressure to the piston the loss of sap was made up by the injected solution. The advantage was that no excess of pressure had to be applied to the interior of the cell, and that after practice it was possible to determine the amount of entering solution quite accurately by controlling the speed at which the hole was closed up. This plan, however, involved some deflation of the cell which is often fatal. If the cell wall is creased or folded the protoplasm is likely to become detached at this point, and this disturbance spreads until death occurs. When the cell is transferred from the capillary to the sea water after injection by the first method described, it is obvious that the moment it leaves the tip there must be a fall in the internal pressure by the escape of liquid through the orifice. Undoubtedly the time of outflow would be very short, both because the internal pressure would be high, and the orifice (which may be regarded as a very short capillary of relatively great diameter) would offer very small resistance to the outflow. Hence we should anticipate a great acceleration of the liquid at the orifice but we should not expect any great scouring of the protoplasm at the circumference for owing to the size of the opening the greatest acceleration would be opposite the center of the orifice, decreasing outward to the circumference. This is in marked contrast to the case previously discussed, where the flow was through a tiny annular orifice. Here owing to the smallness of the opening the greatest acceleration would be opposite the edges. It is probable that most of the material which escaped from the cell when the pressure fell was natural sap; for the injected sap plus  $MnCl_2$  had a greater density and would tend to fall to the bottom of the cell away from the site of the puncture. This could actually be observed in certain preliminary experiments where the pink Co ion was injected. The ideal condition would have been to have released the pressure on the piston at the moment the cell left the capillary. This, however, could not be realized in practice and in consequence the exterior of the cell was subjected to a rain of the toxic solution at this time. However, by holding the cell just above the surface of a large beaker of sea water the length of this exposure was cut down and after injection the cell was removed from the beaker at once and washed in a stream of sea water. This served to rock and rotate the cell, and helped to bring about a thorough mixing of the sap and the slightly heavier toxic solution which was injected. The cell was then

placed in a 50 cc. crystallizing dish containing fresh sea water. This was kept covered to prevent evaporation. Each cell was preserved in a separate crystallizing dish to avoid the effect of exosmosis from other cells.

It was recognized that before healing was complete a certain amount of  $MnCl_2$  might escape from the cell by diffusion through the orifice. In order to minimize any effect that this might have on the exterior of the cell, the sea water was changed a few hours after injection and then daily until the cell was healed.

To apply the toxic solution to the exterior the cells were placed in sea water containing varying concentrations of  $MnCl_2$ , 1 cell to a dish. Weighed amounts of  $MnCl_2 \cdot 2H_2O$  were added to sea water, but owing to the uncertain water content, the solutions so made up were analyzed for manganese.

It is plain that when the solution is injected into the cell we must consider not only the action of the toxic solution of the layer *Y* but also the effect of the puncture on the protoplasm. An attempt was made to eliminate the effect of stabbing by trying to determine the percentage of cells dying as the result of the stab alone but this was abandoned in favor of the plan of impaling all cells. All the cells of group *B* before being placed in the sea water containing  $MnCl_2$  were injected with artificial sap containing no Mn. The possibility of the diffusion of  $MnCl_2$  through the orifice into such cells must be considered. We might have permitted each cell to heal in sea water before placing it in the toxic sea water. But it was felt that this would give an advantage to these cells in comparison with those of group *A*, which were exposed to the Mn from the moment of injection.

Our experiments were carried out in two series, the following routine being observed. A few cells were injected with sap containing  $MnCl_2$ , washed, and placed in separate vessels of sea water, then the syringe was washed out with distilled water and finally with artificial sap. Then the same number of cells were injected with artificial sap,<sup>9</sup> washed, and transferred to separate vessels of sea water containing  $MnCl_2$ . Finally a smaller but proportional number of cells were injected with the sap containing no  $MnCl_2$ , and these were placed, after washing, in sea water to serve as controls. This process was repeated, until the required number of cells for the series had been obtained. By proceeding in this way we made certain that the cells in each group were injected under exactly comparable conditions.<sup>9</sup>

It was not possible to control, except very roughly, the concentration of  $MnCl_2$  applied to the interior of the cell. A small measure of control could be exercised by varying the concentration of the  $MnCl_2$  in the injected sap. In practice two solutions were used, containing respectively about 0.05 N and 0.25 N  $MnCl_2$ . These concentrations represented a compromise. When a solution 0.01 N was injected into 25 cells 18 of them lived for more than 2 weeks without sign of injury. At the end of this period the experiment was terminated. On analysis only 6

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<sup>9</sup> The needles were of course very fragile and when one broke during the stabbing the cell was rejected.

cells showed more than a trace of  $\text{MnCl}_2$  present, while in two cases no Mn was detected. On the other hand, when a solution containing 0.1 N  $\text{MnCl}_2$  was injected into a group of 9 cells, 8 of them failed to heal and within 48 hours the protoplasm had detached itself from the cellulose wall.

During the first 2 days after injection all the cells were examined carefully for signs of healing. In this respect the behavior of the control cells was of great interest. Many of these showed definite signs of recovery in 6 hours, and in nearly all cases healing was complete (as far as the eye could detect) within 24 to 48 hours. The visible signs of healing are the deposit of a black material in the puncture and the regaining of full turgidity.<sup>10</sup> Most of these cells continued to live indefinitely. A group of such healed cells compared with a group of unpunctured cells kept under similar conditions failed to show an appreciably higher mortality in 20 days. The effect of impalement is apparently shown at once, and if impaled cells heal they will survive almost as well as unpunctured cells. This conclusion is in line with our previous experiments<sup>8</sup> on potential difference in which cells were impaled and left on capillaries in sea water for periods up to a month, without suffering any apparent injury.

We believe that our procedure eliminated the effect of the impalement. All cells which persistently remained soft were rejected, and all cells which showed complete or even partial healing were kept (by partial healing we mean that a few cells showed the deposition of the black material in the puncture but died before they had completely recovered their turgidity).

It was observed that in the presence of Mn a series of changes took place with considerable regularity. Sometime after the sealing of the puncture, the protoplasm showed long thin areas of a lighter color. These widened, and became still lighter or even colorless. This process continued until the protoplasm was without color except for very small dark irregular patches. During these changes the cell retained its usual turgidity. At length it softened abruptly. At this stage the protoplasm either detached itself spontaneously from the cellulose wall or the detachment occurred when the cell was gently rolled between the fingers. These changes took place in both group A and group B when the concentration of the  $\text{MnCl}_2$  was high. At lower concentrations the appearance of the lighter areas was either very much delayed or if they appeared early they spread comparatively slowly. In these cases the protoplasm usually detached itself spontaneously and the "colorless" and "soft" stages were absent. Variations of these main types of behavior were also observed. It was therefore necessary to select a stage in the protoplasmic changes to serve as a reference point in comparing the length of

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<sup>10</sup> About 7 per cent failed to heal. These remained soft and within 24 to 72 hours showed a definite detachment of the protoplasm from the cellulose wall. In many of these cases the protoplasmic structure was greatly altered, and in others on gentle rolling between thumb and forefinger the protoplasmic layer broke up.

life of the cells. Only two points seemed suitable, namely: the first appearance of the light streaks, and the detachment of the protoplasm. The first of these was less desirable for it was not always possible to distinguish slight irregularities present at the start from the incipient streakiness. The detachment of the protoplasm was therefore selected as our reference point. There was no detectable diffusion of  $Mn^{++}$  or  $SO_4^{--}$  into the cell (and no diffusion of  $Mn^{++}$  from the cell) at any stage preceding the detachment of the protoplasm. A few hours after this had occurred, however, sufficient diffusion of these ions across the membrane had taken place to be readily detected. It would therefore seem as though this is the point at which the protoplasm becomes freely permeable. In the discussion which follows we shall designate cells which have reached this point as "dead."<sup>11</sup>

### *Analyses.*

It was not, of course, possible to tell the exact moment when the protoplasm became freely permeable in cases where its detachment was spontaneous and hence in cells of group *A* it was necessary to take into account  $MnCl_2$  which might have passed out into the sea water by diffusion. In making the analyses, therefore, the sea water was tested for Mn with the delicate reagent potassium periodate. When Mn was detected, care was taken to extract all the sap from the cell. This was done by piercing it with a fairly fine capillary pipette and by applying considerable suction. The external pressure caused the cell to collapse completely, and drove not only all the sap but also the detached protoplasm into the pipette. The sap was blown from the pipette into a small glass-stoppered bottle and weighed. A certain amount of inaccuracy was therefore introduced into the analysis since the weight would be too great by the weight of the protoplasm. On the other hand since some of the Mn had passed out into the sea water the weight of sap from this cause was too little. We cannot accurately estimate these errors which tend to compensate each other, nevertheless it does not seem that they could have affected the final results very seriously. After the sap had been weighed it was added to the sea water in which the cell had stood and the solution was evaporated with 0.5 cc. of concentrated  $HNO_3$ , to oxidize the protoplasm, and 0.5 cc. of concentrated  $H_2SO_4$ , to change all the chlorides to sulfates. As an extra precaution, because of the large amount of chloride present, a second evaporation with  $H_2SO_4$  was performed. The Mn was then oxidized according to the well known bismuthate method, and compared with standards containing known amounts of Mn.<sup>12</sup> In the group *B* analyses, where no appreciable amounts of Mn were detected in the sea water bathing the cells, the sea water was not included for the analysis, and since it was not necessary to empty the cell com-

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<sup>11</sup> See also page 210.

<sup>12</sup> For details of the analysis see Standard methods for the examination of water and sewage, American Public Health Association, Boston, 5th edition, 51.

pletely, sap free from protoplasm was obtained for analysis. We have not been able to detect in these two sets of group *B* results any systematic variation comparable with the natural variation of the cells themselves.

#### DISCUSSION.

The effect of the treatment is shown in Fig. 3. For group *A* (Mn applied externally) the figures were obtained by averaging the length of life of all the cells (usually 18) at each concentration. For

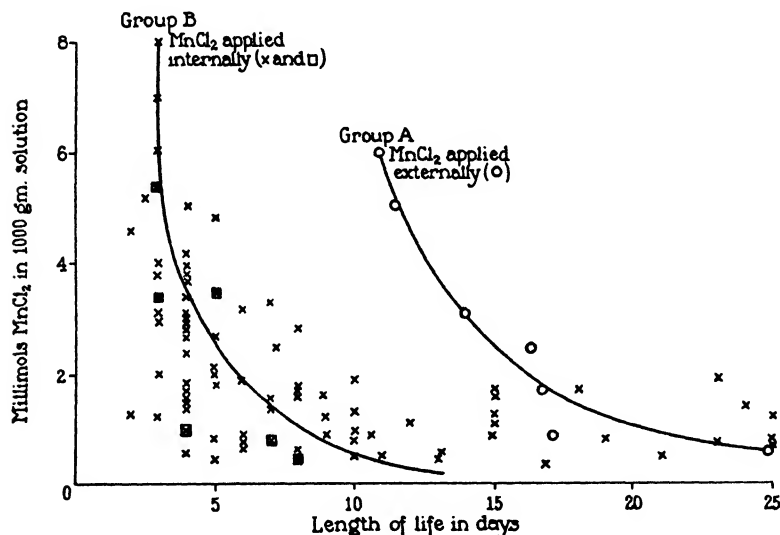


FIG. 3. Duration of life. The points for group *A* are obtained by averaging the length of life at each concentration. Those for group *B* are separate determinations of individual cells, the curve being drawn free-hand without attempting anything more than a rough fit.

group *B* (Mn applied internally) the length of life of each cell was plotted and a curve drawn free-hand without any attempt to do more than make a rough fit. The scattering of the points at low concentrations is to be expected since in general the application of dilute solutions of toxic substances gives very irregular results. (In general the controls lived more than a month after being stabbed.)

It is obvious that the cells of group *A* lived about twice as long as



those of group *B*. The difference might have been a little greater if the amount of Mn available for combination with the protoplasm had been the same in both cases; but where the Mn was applied externally at the same concentration as in the sap the external volume of solution was greater (10 to 15 cc. for each cell). Hence if the protoplasm combined with most of the Mn there would be more available in the latter case but it is not probable that more than a small fraction of the Mn was taken up by the protoplasm.<sup>13</sup>

Earlier in this paper we spoke of the possibility of the diffusion of  $\text{MnCl}_2$  from the sea water into the cell before healing was complete. This might tend to reduce the average life of the cells by subjecting *Y* as well as *X* to the action of the toxic agent. This would tend to bring the curves closer together. Hence any error involved would not invalidate the conclusion that the injection of the  $\text{MnCl}_2$  produces death more quickly than application to the exterior.

Hydrolysis<sup>14</sup> of  $\text{MnCl}_2$  might affect the results by increasing the acidity but our tests indicate that this effect is negligible.

If we take the curves as drawn we see that, for example, when 0.0003 *M*  $\text{MnCl}_2$  is applied internally the cells live about 12 days; to shorten the life of the cell to the same extent by an external application would require about 0.0045 *M*, a concentration more than 10 times as great.

The simplest interpretation might be to regard the inner layer, *Y*, as more sensitive than the outer, *X*, and to suppose that as soon as the inner layer is sufficiently altered to become permeable to  $\text{MnCl}_2$  the latter diffuses through *Y* and attacks *X*. An attack from the outside would be slower because of the greater resistance of *X*.

An alternative assumption might be that *X* is permeable to the toxic agent and *Y* is not, and that the layer which is permeable is not

<sup>13</sup> It is of course possible that some Mn may combine with the cell wall in such a way as to be removed from the sphere of action but it does not seem probable that this is a factor of importance, especially as each cell was placed in a separate dish containing 10 to 15 cc. of solution.

<sup>14</sup> The hydrolysis of  $\text{MnCl}_2$  appears to have been measured only once. Thus Kullgren (*Z. physik. Chem.*, 1913, lxxv, 473; see Landolt-Bornstein, *Physikalisch-chemische Tabellen*, Berlin, 5th edition, 1923, ii, 1170) found by the sugar inversion method that a 0.25 *M*  $\text{MnCl}_2$  solution at 100°C. was hydrolyzed only 0.0017 per cent.

injured by the  $MnCl_2$  which diffuses through it so that when the toxic solution is injected  $Y$  will be attacked at once, but  $X$  will not be injured until after the alteration of  $Y$ .<sup>15</sup> When the toxic solution is applied to the outside of the cell  $Y$  will be subject to attack only after the toxic substance has diffused through  $X$ .

Even if there are differences of permeability it is probable that we also have differences of sensitivity as well to deal with. The assumption that  $X$  and  $Y$  are unlike is in harmony with our previous work on potential differences across the protoplasm of *Valonia*<sup>16,17</sup> and with experiments on the marine alga *Griffithsia*<sup>18</sup> and with the investigations of de Vries<sup>19</sup> in so far as they may be interpreted to mean that the inner and outer surfaces do not act alike. But the statement of Höber<sup>20</sup> that the inner and outer surfaces of the protoplasm may be unlike seems to apply rather to a difference between the inner and outer surfaces of  $X$ .

It is probably not worth while to attempt to compare our results with those of Chambers and his coworkers who have in some cases found much less toxicity when a substance is injected into *Amaba* than when applied to the outside,<sup>21</sup> since in these cases the toxic substance was not injected into a pre-existing vacuole as in the case of *Valonia* where the mechanism involved may be quite different.

<sup>15</sup> Throughout this part of the discussion the destruction of either layer is considered to be equivalent to death.

<sup>16</sup> Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, xi, 193.

<sup>17</sup> Experimental evidence seems to indicate that  $X$  is actually permeable to certain ions, while  $Y$  may not be.

<sup>18</sup> Osterhout, W. J. V., *Science*, 1913, xxxviii, 408.

<sup>19</sup> de Vries, H., *Jahrb. wissenschaft. Bot.*, 1885, xvi, 465. See also Küster, E., *Ber. bot. Ges.*, 1909, xxvii, 589; *Arch. Entwicklungsmechn. Organ.*, 1910, xxx, pt. 1, 351 (Festschrift für Wilhelm Roux); *Z. Bot.*, 1910, ii, 689.

<sup>20</sup> Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 6th edition, 1926, 732.

<sup>21</sup> A particularly striking case is that of picric acid, as described by Pollack and Howland (Pollack, H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 145. Howland, R. B., and Pollack, H., *Proc. Soc. Exp. Biol., and Med.*, 1927, xxv, 221).

## SUMMARY.

When  $\text{MnCl}_2$  is injected into the cells of *Valonia macrophysa* they live only about half as long as when the same concentration is applied to the exterior of the cell. This is due to toxic action and not to the mechanical disturbance accompanying the injection (since all cells were stabbed in the same manner by the capillary).

A variety of explanations are suggested, all of which involve a difference between the inner and outer layers of the protoplasm.



## THE DEATH WAVE IN NITELLA.

### II. APPLICATIONS OF UNLIKE SOLUTIONS.

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(Accepted for publication, June 27, 1928.)

Previous experiments lead us to expect that protoplasm will give a negative current of injury when in contact with dilute solutions and that the opposite will be true with concentrated solutions. This should enable us to predict what will happen when a cell is brought simultaneously in contact with both kinds of solutions. It is shown in this paper that such prediction is possible.

These predictions involve the assumption that the protoplasm is composed of layers, *i.e.* an outer (*X*) and an inner (*Y*) both of which are probably non-aqueous and between these an aqueous layer (*W*). As previously explained<sup>1</sup> the more rapid alteration of the outer layer produces a positive current of injury and the more rapid alteration of the inner gives a negative current of injury. The latter is observed when the protoplasm is in contact externally with a solution less "effective"<sup>2</sup> than sap (*e.g.* 0.001 M KCl); the former occurs with solutions more effective than sap (*e.g.* 0.1 M KCl.) Hence if we apply 0.001 M KCl to the cell the outer layer (*X*) will be in contact with a less effective solution than the inner (*Y*) (which is in contact with sap).

If we arrange an experiment as in Fig. 1 with 0.001 M KCl at *A* and 0.1 M KCl at *C*,<sup>3</sup> and cut at *Z* we shall expect that, as previously described,<sup>1</sup> the death wave which starts at *Z* will pass rapidly down the cell to *C*. At *A*, where the protoplasm is in contact with 0.001 M KCl, we shall expect the inner layer (*Y*) to go first so that the protoplasm will at first become more negative<sup>1</sup> but at *C*, where it is in contact with 0.1 M

<sup>1</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, xii, 167.

<sup>2</sup> The more effective solution is the one which causes a more rapid alteration.

<sup>3</sup> The technique has been fully described in previous papers. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 391, 417, 673; 1928-29, xii, 167.

KCl, the alteration of  $X$  will be more rapid than that of  $Y$  and in consequence the protoplasm will first become more positive.

That this is really so can be shown by arranging an experiment as in Fig. 2, killing  $B$  with 0.01 M KCl saturated with chloroform and leading off from  $A$  (in contact with 0.001 M KCl) to  $B$  and from  $C$  (in contact with 0.1 M KCl) to  $B$ : on cutting at  $Z$  we obtain approximately the "true" curves of  $A$  and  $C$  (*i.e.* the curves obtained when the value at  $B$  is zero), as shown in Fig. 3 *a*.<sup>4</sup> If we lead off from  $A$  to  $C$  we obtain

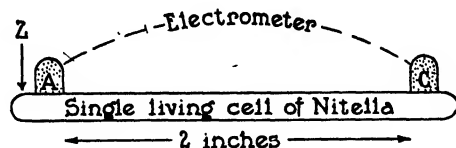


FIG. 1. Diagram to show the arrangement of certain experiments. At  $A$  and  $C$  are flowing contacts, or pieces of cotton soaked in solutions, or cups containing solution.

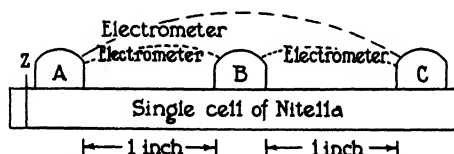
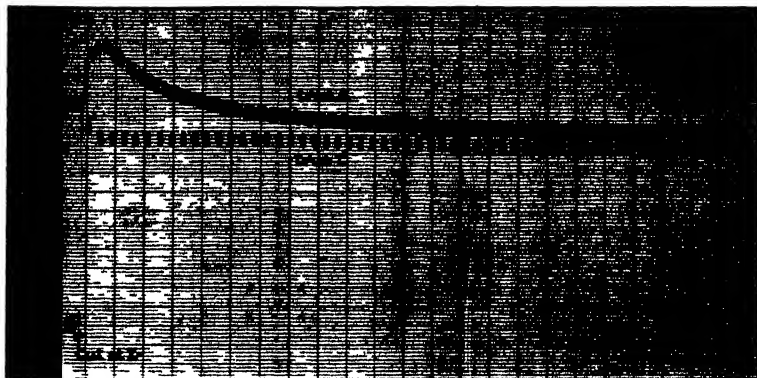
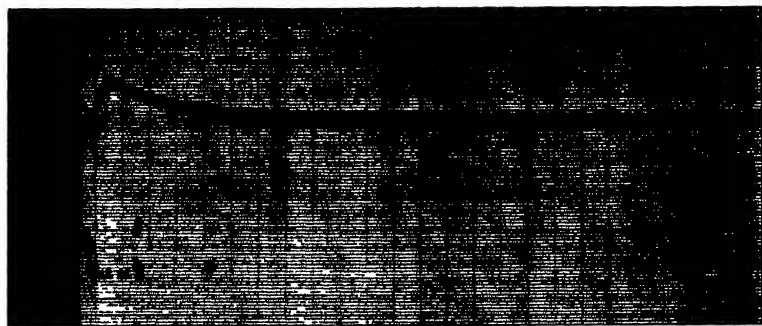


FIG. 2. As in Fig. 1 with an additional contact at  $B$  which is connected with  $A$  and with  $C$ .

approximately the difference between the "true" curves of  $A$  and  $C$ , as shown in Fig. 3 *b*.

The result corresponds to our prediction, *i.e.* at  $A$  (which is in con-

<sup>4</sup> The values at  $A$  will be more positive than they should be by a small but definite amount because of an effect due to the cell wall (see Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673) which makes  $A$  appear more positive. This effect will remain constant until sap diffusing out at  $B$  raises the concentration of the solution imbibing the cell wall; the effect of this will be to make  $A$  appear still more positive but in very short experiments it may be neglected. What is said of  $A$  applies equally to  $C$  (in contact with 0.1 M KCl) except that the cell wall effect will make  $C$  appear somewhat more negative than it should be at the start and the coming out of sap at  $B$  will decrease this effect. These effects are small and may be neglected for our present purpose.

FIG. 3 *a*.FIG. 3 *b*.

FIGS. 3 *a* and *b*. Photographic records showing potential differences, the experiment being arranged as in Fig. 2 with 0.001 M KCl at *A*, 0.01 M KCl at *B*, and 0.1 M KCl at *C*. The record starts after *B* has been killed (with 0.01 M KCl saturated with chloroform) so that *A* and *C* show their "true" values. On cutting at *Z* we see (in Fig. 3 *a*) that *A* becomes negative and then approaches zero while *C* becomes positive and then approaches zero. Curves *A* and *C* are recorded on one instrument while the *A* to *C* curve is simultaneously recorded on another (Fig. 3 *b*): this curve is approximately equal to the difference between the "true" curves of *A* and *C*.

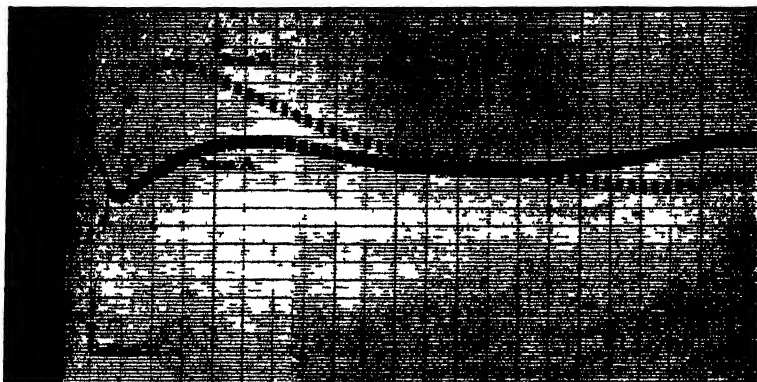


FIG 4 a.

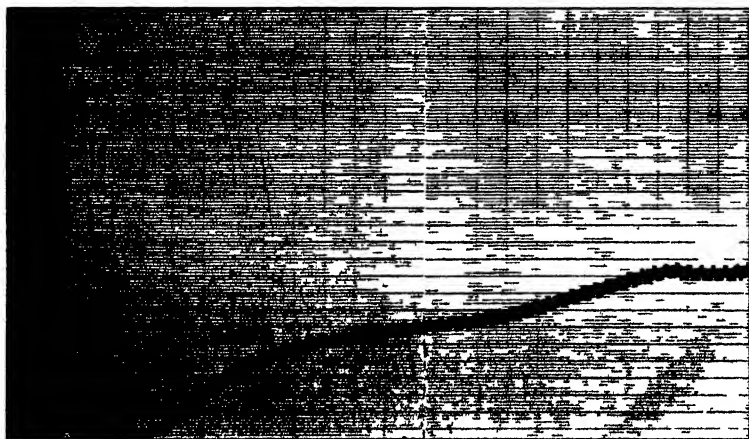


FIG. 4 b

FIGS 4 a and b Photographic records showing potential differences, the experiment being arranged as in Fig 2 with 0.1 M KCl at A, 0.001 M KCl at B, and 0.01 M KCl at C. The record starts after C has been killed (with 0.01 M KCl saturated with chloroform) so that A and B show their "true" values. On cutting at Z we see (in Fig 4 a) that A becomes positive and then approaches zero while B becomes negative and then approaches zero. Curves A and B are recorded on one instrument while the A to B curve is simultaneously recorded on another (Fig. 4 b): this curve is approximately equal to the difference between the "true" curves of A and B.



tact with a solution less effective than sap) the protoplasm first becomes more negative as the result of the cut, indicating that the layer *Y* goes first; at *C* (which is in contact with a solution more effective than sap) the protoplasm first becomes more positive, indicating that the layer *X* goes first.

It is desirable to supplement this experiment by placing 0.1  $\text{m}$  KCl at *A*, 0.001  $\text{m}$  KCl at *B*, and then killing *C*: the result is shown in

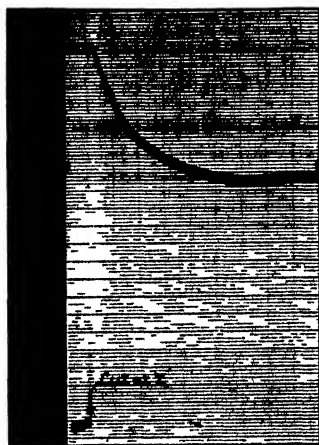


FIG. 5.

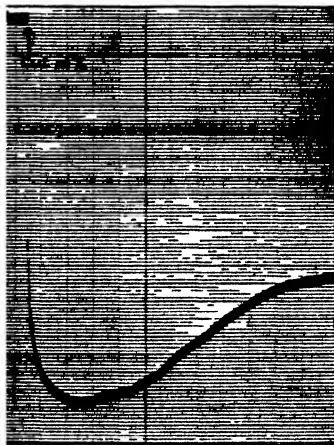


FIG. 6.

FIG. 5. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001  $\text{m}$  KCl at *A* and 0.1  $\text{m}$  KCl at *C*. On cutting at *Z* we obtain a curve resembling that in Fig. 3 *b*.

FIG. 6. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.1  $\text{m}$  KCl at *A* and 0.001  $\text{m}$  KCl at *C*. On cutting at *Z* we obtain a curve similar to that in Fig. 4 *b*

Figs. 4 *a* and 4 *b* (since *C* is killed we obtain the "true" curves of *A* and *B*). The result is again in accordance with prediction.

It may be thought that killing a spot may alter the condition of the cell and so affect the curves. We have accordingly performed experiments (arranged as in Fig. 1) in which the killing is omitted: these are illustrated by Figs. 5 and 6 which resemble Figs. 3 *b* and 4 *b* sufficiently to indicate that the killing of a single spot makes no essential

difference. In all these cases we are dealing with the difference between two spots, *A* and *C*, or *A* and *B*, which are affected in turn by a death wave which starts from *Z* and travels down the cell.

An interesting experiment is shown in Fig. 8. The experiment was arranged as in Fig. 7 with 0.1 *M* KCl at *A*, 0.001 *M* KCl at *B*, and 0.05

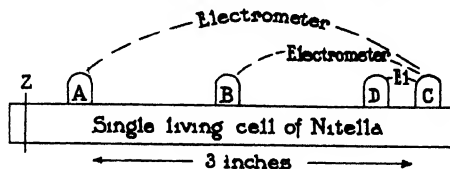


FIG. 7. As in Fig. 1 with additional contacts at *B* and *D*.

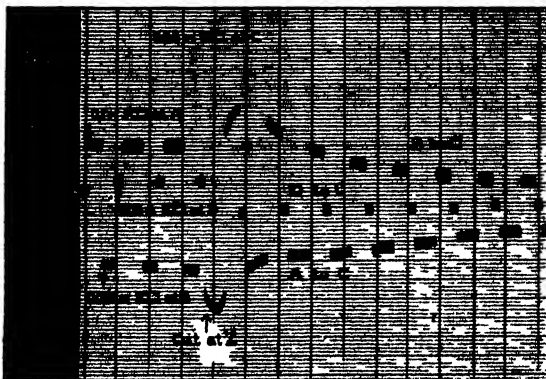


FIG. 8. Photographic record of potential differences, the experiment being arranged as in Fig. 7 with 0.1 *M* KCl at *A*, 0.001 *M* KCl at *B*, 0.05 *M* KCl at *D* and at *C*. On cutting at *Z* we see that *A* becomes positive and then approaches zero, *B* becomes negative and then approaches zero; *D* shows little change because it is in this case very near *C* and is in contact with the same solution: it becomes slightly positive (as expected) and then approaches zero.

*M* KCl at *D* and at *C*. At the start *A* is negative and *B* positive due to the concentration effect.<sup>5</sup> On cutting at *Z*, *A* changes from negative to positive and *B* from positive to negative. *D* shows little change

<sup>5</sup> This is discussed in Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.

because it is very close to *C* and is in contact with the same solution (its curve is in accordance with expectation<sup>1</sup>).

The fact that *B* becomes negative on cutting is an argument against the theory mentioned in a previous paper<sup>6</sup> that injury causes an instantaneous exit of sap which might account for the results without the necessity of assuming the presence of layers in the protoplasm. The coming out of sap at *B* could not in any case do more than bring *B* to the same condition as *C* and so reduce the potential difference between them to zero; it could not make *B* negative as is the case here (since sap in these experiments acts like 0.05 M KCl).

A great variety of combinations were tried which need not be described in detail since the results were in every case in accord with the theory previously developed. We may conclude that the theory serves a useful purpose in bringing all the facts under a single viewpoint and in enabling us to predict the behavior of the cell under a great variety of conditions.

#### SUMMARY.

The hypothesis of protoplasmic layers enables us to predict the bioelectrical behavior of the cell under a great variety of conditions. It is shown in the present paper that this is clearly the case when a death wave passes through different points in contact with unlike solutions.

<sup>6</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.



## THE PENETRATION OF STRONG ELECTROLYTES.

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This paper deals primarily with the penetration of strong electrolytes. It touches incidentally on the problem of flotation since in some cases flotation was used as a criterion of penetration: as examples of this we may cite experiments in which  $\text{NH}_4\text{Cl}$  and  $\text{CsCl}$  were added to sea water.

Striking results were obtained by adding small amounts of 0.6 M  $\text{NH}_4\text{Cl}$  to sea water. The specific gravity of the sea water was very slightly decreased and at first the cells remained at the bottom but after a time they began to float because  $\text{NH}_4\text{Cl}$  penetrated and lowered the specific gravity of the sap so much that the cells rose to the surface: here they continued to live and grow indefinitely. (It is probable that  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  penetrates and is subsequently changed to  $\text{NH}_4\text{Cl}$ ¹).

When the concentration of  $\text{NH}_4\text{Cl}$  in the sea water was 0.005 M² enough  $\text{NH}_4\text{Cl}$  penetrated in 10 days³ to cause the cells to float. Examination showed that the specific gravity of the sap was then lower than that of the sea water (see Table I); it was analyzed to determine the proportions of salts present and it was found that artificial solutions made up in these proportions had practically the same specific gravity as that of the samples of sap.⁴ Obviously therefore the penetration of  $\text{NH}_4\text{Cl}$  is sufficient to account for the change in specific gravity (see Table I).

¹ Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 234.

² This did not increase the osmotic pressure of the sea water more than 0.1 per cent.

³ These cells were placed in bottles and submerged in the ocean where growth continued.

⁴ The composition of these samples will be discussed in a later paper. The specific gravity of the sap in some cases fell (in the course of 22 days) to 1.0195 (taking water at 25°C. as 1.000).

We may surmise that if the ocean contained a little more  $\text{NH}_4\text{Cl}$  many organisms might float which do not now do so. Such a change has great biological importance since it profoundly alters the environment (and the distribution) of an organism to bring it from the bottom to the surface of the water. Some organisms such as *Halicystis*<sup>5</sup> float by excluding certain substances, especially sulfates. It would be exceedingly interesting to know how many mechanisms are employed for flotation and how it can be experimentally produced or suppressed.

Experiments with flotation produced by increasing the specific gravity of the sea water by adding  $\text{CsCl}$  also gave striking results. When 1 volume of  $\text{CsCl}$  0.6 M was added to 9 volumes of sea water the halide content and osmotic pressure remained about the same as

TABLE I.

Specific gravity (at 25°C taking water at 25°C as 1 000)		Remarks
Sea water.	1 02250	Cells sink
9 volumes sea water + 1 volume $\text{CsCl}$ 0.6 M	1 02740	Cells float
Normal sap	1 02250	Cells sink
Sea water containing 0.005 M $\text{NH}_4\text{Cl}$	1 02245	Cells sink
Sap of cells which had been 22 days in sea water containing 0.005 M $\text{NH}_4\text{Cl}$	1 01950	Cells float

in ordinary sea water; the specific gravity rose from 1.0225 (ordinary sea water) to 1.0274. Cells sink<sup>6</sup> in ordinary sea water but after the addition of  $\text{CsCl}$  they float (see Table I). It is evident that they would sink if sufficient  $\text{CsCl}$  should penetrate but this did not happen although the cells lived for more than a year: the only cells that sank during this time were a few that died.<sup>7</sup> This experiment recalls that of Loeb<sup>8</sup> on *Fundulus* but differs in that the external medium was not toxic nor of higher osmotic pressure than the sea water.

<sup>5</sup> This was formerly called *Valonia ventricosa*: for an account of its flotation see Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, vii, 633.

<sup>6</sup> The specific gravity of the sap is normally about the same as that of sea water but the weight of the cellulose wall and of the protoplasm causes the cells to sink.

<sup>7</sup> The cells were kept in finger bowls covered by plates of glass near a north window.

<sup>8</sup> Loeb, J., *Biochem. Z.*, 1912, xlvii, 127.

The experiment was discontinued after 13 months and the sap was extracted by piercing the cells with a sharp glass capillary (after rinsing the outside for a few seconds with distilled water and drying lightly with filter paper). It was found that the halide concentration of the medium (sea water containing CsCl) had risen, as the result of slow evaporation, from 0.564 M to 0.858 M: that of the sap<sup>9</sup> had risen to 0.885 M. The specific gravity of the medium had risen from 1.0274 to 1.0473 and that of the sap<sup>9</sup> to 1.0410.

We are indebted to Professor G. P. Baxter for making a spectroscopic examination of the sap: this showed a very small amount of Cs (probably less than 0.1 per cent); this might have been due to injury of some of the cells, or to contamination of the sap during its extraction. In any case it is evident that little or no Cs penetrated the normal cells during a period of over a year.

A similar experiment was made with RbCl by adding 1 volume of RbCl 0.6 M to 4 volumes of sea water, making the concentration of RbCl 0.12 M. This produced scarcely any change in freezing point depression but raised the specific gravity from 1.0225 to 1.0271. Some of the cells sank after a day and appeared normal but others floated for a few days, after which the experiment was discontinued.

It is therefore evident that there is little or no penetration of CsCl but it seems probable that there is more penetration of RbCl. We know that KCl and NaCl penetrate (either as such or perhaps as KOH and NaOH)<sup>1</sup> since they are found in the vacuole, but our experiments show that such penetration is very slow.

It would seem that K penetrates most rapidly and Cs least rapidly, the probable order being  $K > Na > Rb > Cs$ . Li also penetrates but its position in the series is doubtful. It may be that the alkalis<sup>10</sup> penetrate the protoplasm as undissociated molecules (*e.g.* as hydrates)<sup>11</sup> whose solubility in the protoplasm may increase in the order given. Höber<sup>12</sup> has recently suggested that the order of penetration is that

<sup>9</sup> The normal halide content of the sap is about 0.028 M greater than that of the surrounding sea water and its specific gravity not far from that of sea water.

<sup>10</sup> Nothing is said in this connection regarding  $NH_4^+$  since it probably penetrates to a considerable extent as  $NH_3$ .

<sup>11</sup> See Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 234.

<sup>12</sup> Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, ccxix, 260.

of the ionic radius but this would require that  $\text{Cs}^+$  should enter more readily than  $\text{K}^+$ .

It should be borne in mind that these ideas cannot be applied generally since in *Halicystis* (formerly called *Valonia ventricosa*<sup>13</sup>) the composition of the sap suggests that Na penetrates more rapidly than K.

It may be of interest to consider the absolute amounts taken up. The best data are those on the absorption of K, Na, and Cl under favorable circumstances. Here, as in other experiments on strong electrolytes, conditions which favor growth favor penetration (as is to be expected since the relation of the salt content of the sap to that of the sea water remains nearly constant). It might be expected that even when no growth occurs cations might penetrate as the result of exchange but our experiments (e.g. attempting to exchange K for Na by increasing the K content of the sea water) indicate that such processes must be very slow. Cells placed in bottles and suspended in the ocean (from July 1 to September 5) increased in weight at the rate of about 1 per cent per day.<sup>14</sup> If we take as an average representative of these cells a prolate spheroid 1.3 cm.  $\times$  0.78 cm. (this measurement does not include the cell wall) we should have for the surface area of each cell  $2\pi [b^2 + (ab \div e) \sin^{-1}e]$  where  $a$  = one half the major axis (= 0.65),  $b$  = one half the minor axis (= 0.39), and  $e$ , the eccentricity, is 0.8 (from the equation  $e^2 = (a^2 - b^2) \div a^2$ ). This gives for the surface 2.8 sq. cm. The volume is  $(4 \div 3)\pi ab^2 = 0.41$  cc.

Since the concentration of Cl in the sap is about 0.6 M and the concentration of K<sup>15</sup> is 86.2 per cent of this, 1 liter of sap contains  $(0.6) \times (0.862) = 0.5172$  mol of K, and one cell has approximately  $(0.41 \div 1000) 0.52 = 0.000213$  mol of K and takes up per day 1 per cent of this or  $0.00000213 = 213 \times 10^{-8}$ . Since the area of the cell is 2.8 sq. cm. the amount taken up per day per sq. cm. is  $(213 \times 10^{-8}) \div 2.8 = 76 \times 10^{-8}$ , or  $(76 \times 10^{-8}) \div 24 = 3.17 \times 10^{-8}$  mols of K per hour per sq. cm. The intake of Na is this amount multiplied<sup>16</sup> by

<sup>13</sup> Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, vii, 633.

<sup>14</sup> For measurements of growth see Brooks, M. M., *Am. J. Bot.*, 1925, xii, 617.

<sup>15</sup> This was determined by the perchlorate method.

<sup>16</sup> The molar proportion of Na to K in the sap is  $15.1 \div 86.2 = 0.17$ .



0.17 or  $0.55 \times 10^{-8}$  mol of Na per hour per sq. cm.: that of Cl is equal to the sum of K and Na or  $3.7 \times 10^{-8}$ .

It would be interesting to compare these figures with those of Northrop<sup>17</sup> for diffusion through a collodion membrane but it would be necessary to know the pressure which drives K and Na into the cell and we are not able to calculate this with certainty. It may be noted that K moves into the *Valonia* cell against the concentration gradient (the concentration of K is about 40 times as great inside the cell as outside) but Na moves with the concentration gradient (the concentration of Na is nearly 6 times as great outside as inside).

Some experiments with anions may be mentioned here. Cells were placed in a mixture of 3 volumes of sea water + 1 volume of NaI 0.6 M where they floated. After 24 days (under conditions favorable for growth) all the cells still floated except those that had died: the iodide in the sap of the living cells was 0.045 M and in the sea water 0.150 M, but when the growth of the cell is taken into consideration it appears that little or no Cl came out of the cell during the absorption of I.

The case seems to be similar with the intake of Br, at least qualitatively. When 3 volumes 0.6 M NaBr are added to 1 volume of sea water so that the cells float, they sink much sooner when conditions are favorable for growth, indicating that Br is taken up during the process of growth.

A recent paper by Höber and Höber<sup>12</sup> states that considerable bromide penetrates in 7 hours. This is more rapid penetration than occurs in our material unless the cells are injured (we determined Br by the method they employed).

We may conclude that in general the penetration of strong electrolytes into *Valonia* is very slow unless the cells are injured.<sup>18</sup> There

<sup>17</sup> Northrop, J. H., *J. Gen. Physiol.*, 1927-28, xi, 233.

<sup>18</sup> In some cases there is visible temporary injury (as evidenced by the disarrangement of chloroplasts when viewed under the microscope) which is followed by recovery: such experiments are rejected. The best criteria of injury are the microscopic appearance, the turgidity, the absence of disturbance to the protoplasm when the cell is rolled between the fingers or bounced lightly on a table, and the presence of sulfate in the sap. In addition cells should be transferred to sea water and kept under observation but this may tell us nothing in regard to temporary injury during the experiment. Cf. Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147.

appears to be slow penetration with Li,  $\text{BrO}_3$ ,  $\text{IO}_3$ , and selenite, but other substances show much less penetration, *e.g.*  $\text{SCN}$ , ferricyanide, ferrocyanide, formate, salicylate, tungstate, selenate,  $\text{NO}_3$ ,  $\text{SO}_3$ , Sb-oxide, glycerophosphate, and many heavy metals and the alkaline earths.

On the other hand our experiments and those of others show that certain weak electrolytes enter *Valonia* more rapidly, *e.g.*,  $\text{NH}_3$ ,<sup>19</sup>  $\text{H}_2\text{S}$ ,<sup>20</sup>  $\text{CO}_2$ ,<sup>20</sup> and others.

We might explain these facts on the ground that the protoplasmic surfaces consist of non-aqueous layers<sup>21</sup> through which electrolytes pass for the most part in the form of undissociated molecules. We may imagine that ions striking the outer surface of the protoplasm unite to form molecules and as such pass through the non-aqueous surface layer only to dissociate again on reaching an aqueous phase.<sup>1</sup>

If ions penetrate as such it may be by exchange of those of the same sign going in opposite directions<sup>22</sup> or by the entrance of ion groups (*e.g.*  $\text{K}^+$  and  $\text{OH}^-$ ) formed at the surface by collision of ions of opposite sign which then penetrate together. It would seem that such pene-

<sup>19</sup> For experiments on penetration of  $\text{NH}_3$  into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 2074. For experiments on *Nitella* see Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 235. For experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1910, lx, 305. Harvey, E. N., *Carnegie Institution of Washington, Pub. No. 183*, 1914, 131. Jacobs, M. H., *The Harvey Lectures*, 1926-27, 146.

<sup>20</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1925, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, ix, 255. For the results of other workers on weak acids see Loeb, J., *Biochem. Z.*, 1909, xv, 254; 1910, xxiii, 95; *Arch. ges. Physiol.*, 1897-98, lxi, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization, Chicago, 1913, 143; *J. Gen. Physiol.*, 1922-23, v, 231. Harvey E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463; *Carnegie Institution of Washington, Pub. No. 212*, 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922-23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457; *Biol. Bull.*, 1922, xlii, 14. Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 1449, 1470. Beerman, H., *J. Exp. Zool.*, 1924-25, xli, 33. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

<sup>21</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, xi, 83.

<sup>22</sup> Doubtless the cell can produce a sufficient number of ions for exchange, *e.g.*  $\text{H}^+$  and  $\text{HCO}_3^-$ . Cf. Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxvi, December.

tration must be small in view of the fact that Dr. Blinks finds the electrical resistance of the protoplasm in *Valonia* to be very high. This is so, for example, when the protoplasm is in contact with  $\text{NH}_4\text{Cl}$  although the rise in the pH value of the sap shows that either  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  penetrates rapidly: presumably if  $\text{NH}_4\text{OH}$  penetrates it does so in the form of undissociated molecules.

In the case of strong electrolytes the penetration of undissociated molecules or of ion pairs would probably be much slower than exchange of ions since it could take place only when ions of opposite sign happened to collide at the surface.

#### SUMMARY.

The entrance of strong electrolytes into *Valonia* is very slow unless the cells are injured. This, together with the very high electrical resistance of the protoplasm, suggests that they may penetrate largely as undissociated molecules formed at the surface of the protoplasm by the collision of ions.

Under favorable circumstances KCl may be absorbed to the extent of  $3 \times 10^{-8}$  mols per hour per sq. cm. of surface together with about 0.17 as much NaCl. Other substances<sup>23</sup> which seem to penetrate to some extent are Li, Rb, Br,  $\text{BrO}_3$ , I,  $\text{IO}_3$ , and selenite.

Little or no penetration<sup>23</sup> is shown by SCN, ferricyanide, ferrocyanide, formate, salicylate, tungstate, seleniate,  $\text{NO}_2$ ,  $\text{SO}_3$ , Sb, glycerophosphate, and many heavy metals and the alkaline earths.

In sea water whose specific gravity had been increased by CsCl cells of *Valonia* floated for over a year and there was little or no penetration of Cs except as the result of injury.

The penetration of  $\text{NH}_4\text{Cl}$  decreases the specific gravity of the sap and causes the cells to float: under these circumstances they live indefinitely. It is probable that  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  penetrates and is subsequently changed to  $\text{NH}_4\text{Cl}$ . It would seem that if the sea contained a little more ammonia this would be a floating organism.

<sup>23</sup> This statement is based on preliminary experiments only.



## SYNERESIS AND SWELLING OF GELATIN.

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(Accepted for publication, June 10, 1928.)

The name syneresis was given by Graham<sup>1</sup> in 1864 to the phenomenon of the breaking up of jellies on long standing or when disturbed. The jelly then, instead of consisting of one homogeneous mass, becomes segregated into solid lumps surrounded by a thin liquid. Syneresis is quite common in jellies or, as they are called, gels of gelatin, agar, starch, etc. It is possible that the separation of serum from clotted blood and the splitting of soured milk into curd and whey may also be classified under the same term. Various theories have been suggested as to the possible cause of syneresis.

Thus Ostwald<sup>2</sup> considers syneresis as simply a separation of phases similar to the separation of phases in a critical fluid mixture. Lloyd<sup>3</sup> explains syneresis of isoelectric gelatin as being due to the absence of soluble gelatin salts, which otherwise keep the network extended by their osmotic pressure. As it will be seen from further discussion, Miss Lloyd's hypothesis is partly true with respect to the absence of syneresis in gelatin in the presence of electrolytes, although there is apparently some confusion as to the meaning of "soluble gelatin salts." With the exception of some recent work by Liepatoff<sup>4</sup> on *geranine* and Mukoyama<sup>5</sup> on *viscose*, very little quantitative work has been done on syneresis in general and of gelatin in particular.

The writer approached the problem of syneresis in gelatin through

<sup>1</sup> Graham, T., Chemical and physical researches (collected papers), Edinburgh 1876, 619.

<sup>2</sup> Ostwald, W., An introduction to theoretical and applied colloid chemistry, New York, 1917, 93.

<sup>3</sup> Lloyd, D. J., *Biochem. J.*, 1920, xiv, 165.

<sup>4</sup> Liepatoff, S., *Kolloid.-Z.*, 1927, xliii, 396.

<sup>5</sup> Mukoyama, T., *Kolloid.-Z.*, 1927, xlii, 79.

the study of the swelling of blocks of dilute gelatin when immersed in water. All of the former studies on swelling of gelatin have dealt with the swelling of dry gelatin or of concentrated gels. In all these cases there is always a gain of water and an increase in size of the gelatin block even at low temperatures. Quite different results are obtained when blocks of isoelectric gelatin of concentrations of less than 10 per cent are immersed in cold water. Instead of gaining water the blocks of gelatin lose water, and the lower the concentration of gelatin the greater is the amount of water lost.

Table I shows the values of swelling at 5°C. of gelatin gels of various concentrations when immersed in M/1000 acetate buffer pH 4.7. The

TABLE I.

*Swelling of Blocks of Gels of Various Gelatin Content at 5°C. in M/1000 Acetate Buffer pH 4.7.*

Electrolyte-free isoelectric gelatin has been used in making up the gels. Gels set for 24 hrs. at 5°C. before they were placed in the buffer solution.

Concentration of gelatin in gm per 100 cc solution ..	3 0	4 0	5 0	6 0	8 0	10 0	12 0	14 0	16 0	20 0
Original weight of block of gel (after setting) .	1 610	1 600	1 505	1 790	1 890	1 847	2 020	2 120	2 090	2 250
Final weight of block at equilibrium (after 20-25 days).....	1 150	1 140	1 130	1 435	1 730	1 845	2 200	2 470	2 570	3 050
Per cent change in weight	-29	-29	-25	-20	-8	0	+9	+17	+23	+36

values are those obtained at equilibrium, which is reached after 20 to 25 days. It is seen that while at concentrations above 10 per cent the gelatin blocks gain in weight, at the lower concentrations there is a continuous drop in weight.

The theories of swelling as developed by Procter, Wilson, Loeb, Northrop and the writer deal with the process of positive swelling of gelatin. These may be summarized as follows:

1. Swelling is mainly a process of osmosis due to a greater concentration of mobile molecules in the block over that of the outside solution in which it is immersed.

2. The molecules causing osmosis may be diffusible ions, as in the case of swelling due to acids, alkalies and some salts, or non-diffusible molecules or groups of molecules as in the swelling of isoelectric gelatin.

3. A block of solid gel behaves like a true elastic body. The swelling is regulated by the elasticity of the gelatin block. At equilibrium the elastic pressure is equal and opposite to the osmotic pressure.

4. When a gelatin sol sets to a gel it is under no elastic strain as long as it is not immersed in water. As soon as it is put into water or salt solution the osmotic forces begin to act against the elasticity of the block causing a strain in the block due to an increase in the bulk. A strain is also brought about in a block of gelatin when water is removed from it by evaporation.

The tendency of a block of a dilute gel to shrink when placed in distilled water indicates that there is still a possible strain in the gel while it sets, which is contrary to the apparent non-strained condition of gels of higher concentration, as demonstrated by Northrop.<sup>6</sup> With the object of finding a possible explanation of this peculiar behavior of dilute gels the writer undertook a detailed study of the "negative swelling" of gelatin under various conditions.

### *Experimental Procedure.*

All the experiments as well as the weighings were done in a refrigerator room kept at a temperature of about 5°C.

Solutions containing various amounts of isoelectric gelatin in distilled water of pH 4.7 or in salt solutions were heated to 50°C. and coated on weighed microscopic slides (1.5 cc. per slide), or 2 cc. were poured into moulds, consisting of short Pyrex glass tubing of about 15 mm. diameter, which were mounted on a clean paraffin block. The gelatin was allowed to set in the refrigerator for about 20 hours. Care was taken to prevent evaporation by keeping the slides in "moist chambers" or stoppering the tubes with rubber stoppers. After the period assigned for setting, the slides or the blocks, on removal from the tubes, were weighed and put into 150 cc. M/1000 acetate buffer pH 4.7 or into other solutions as described later. After various intervals of time the slides or blocks were dried with filter paper and weighed. In drying blocks of dilute gelatin it was found more convenient to use a clean towel instead of filter paper.

The fluid originally used as the outside solution was distilled water. This was brought to pH 4.7 by means of acetic acid.\* But it was soon found that the pH of the water increased slightly after a few days, with the result that the gelatin began

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<sup>6</sup> Northrop, J. H., *J. Gen. Physiol.*, 1926-27, x, 901.

\* Distilled water made up with acetic acid to pH 4.7 is designated throughout this paper as H<sub>2</sub>O pH 4.7.

to gain in weight instead of reaching a constant value. Hence distilled water was replaced by  $M/1000$  acetate buffer pH 4.7.

Fig. 1 shows the effect of two concentrations of acetate buffer pH 4.7 as well as of  $H_2O$  on the negative swelling of 4 per cent isoelectric gelatin coated on slides. During the first few days there was a loss of water in all the gelatin blocks; but afterwards those that were kept in

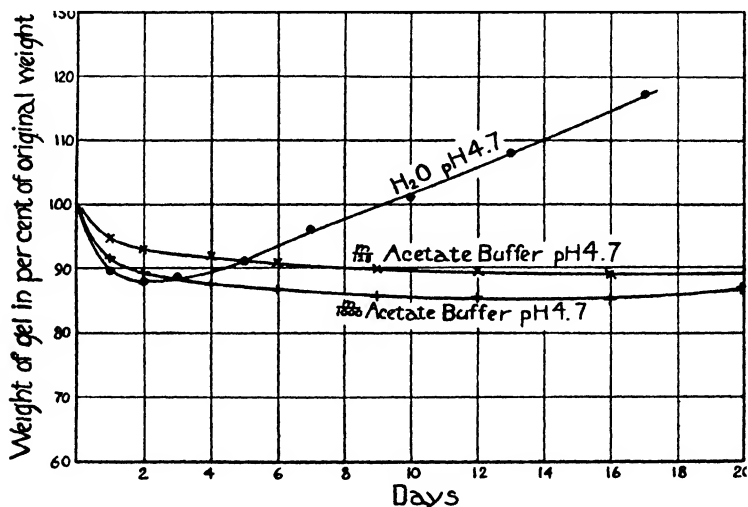


FIG. 1. Effect of a slight change with time in the pH of the outside solution on the change in weight of 4 per cent gels at  $5^{\circ}C$ . The pH of the distilled water became slightly higher than 4.7 on standing.

$H_2O$  began to gain weight continuously, while the others that were put in buffer solution kept on losing weight until the 10th day when an approximate equilibrium was established. After the 20th day the block in  $M/1000$  buffer began to gain slightly in weight but on renewal of the buffer the equilibrium weight was reached again. The experiment also shows that there is less loss in weight of gelatin with the increase in concentration of salt in the surrounding medium. This is simply due to the fact that salts generally increase the swelling of isoelectric gelatin up to a concentration of  $M/2$ .<sup>7</sup>

<sup>7</sup> Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925-28, viii, 317.



*Is the Negative Swelling Due to Solution of the Gelatin?*

A qualitative test on the protein content of the surrounding medium showed that only a trace of the gelatin goes into solution. Quantitative experiments were carried out as follows:

Blocks of 4 per cent of isoelectric gelatin were allowed to remain in large volumes of  $M/1000$  acetate buffer pH 4.7 until the loss in weight became constant. The blocks were then removed, put into weighed evaporating dishes, weighed and then dried in an electric oven at  $100^{\circ}\text{C}.$  for 24 hours. In all cases the dry weight measurements checked with the weight of gelatin as calculated from the loss of

TABLE II.

*Effect of Duration of Setting on the Negative Swelling of 5 Per Cent Isoelectric Gelatin in  $M/1000$  Acetate Buffer pH 4.7.*

Time of setting	0	16 hrs	40 hrs	68 hrs	92 hrs	6 days	8 days
Weight of gelatin after being in moist chamber, in per cent of original weight	100	99.4	100	101	98.5	95	100
Final weight of gelatin in $M/1000$ acetate buffer at equilibrium	87.0	91.0	90.0	86.0	91.0	88.4	90.0

water. Thus it is clear that while some of the gelatin may go into solution, the amount is too insignificant to affect the results. The negative swelling is due to loss of pure solvent.

*Effect of Duration of Setting on the Negative Swelling of Gels.*

A number of weighed slides were coated with 5 per cent isoelectric gelatin. The slides were then reweighed and placed over water in rubber-stoppered glass tubes. Care was taken that the gelatin was not in contact with the water. After various intervals of time the slides were removed, weighed and put into  $M/1000$  buffer pH 4.7. The results are given in Table II.

It is seen that the duration of setting has very little effect on the final loss of water from the gel. In addition the experiment shows that the amount of water lost from the gelatin while kept in a closed moist chamber is very small during the first few days.

*Effect of Drying of Gels.*

A series of slides were coated with 5 per cent isoelectric gelatin solution and then placed for various lengths of time in loosely covered Petri dishes the cover of which had a padding of moist filter paper. This was done in order to prevent too rapid drying of the gels. The slides were afterwards put into  $m/1000$  buffer pH 4.7. Table III shows that the final loss of water is independent of the loss of water due to evaporation. If more water was lost by evaporation than would have been the case had the block been immersed in water, then the gelatin gains water when placed in liquid. In other words, the equilibrium value can be obtained from two directions.

TABLE III.

*Effect of Drying on the Equilibrium of Negative Swelling of 5 Per Cent Isoelectric Gelatin in  $m/1000$  Acetate Buffer pH 4.7.*

Time of drying, in hrs. ....	4	16	44	68	92	116
Weight of gelatin after drying, in per cent of original weight. ....	99.0	97.0	93.0	90.0	87.0	83.0
Final weight of gelatin in $m/1000$ acetate buffer. ....	87.0	87.5	87.5	87.5	88.0	87.0

*Effect of Volume of Outside Solution or Size of Block on the Equilibrium Value of Negative Swelling.*

A 4 per cent solution of isoelectric gelatin was allowed to set for several days in a stoppered flask. No fluid was observed on the surface of the gelatin except for a few drops of condensed vapor on the sides of the flask. A lump of about 10 gm. of gel was then removed by means of a spatula and placed in a weighed porcelain Gooch crucible and its weight determined. The gel was then mashed up by means of the spatula. Fluid immediately began to ooze from the gelatin. Also fluid appeared in the cavity in the stock of gelatin in the flask. The crucible was left to drain overnight on a Gooch funnel. The weight of the gelatin next morning was found to be 82 per cent of the original. The crucible was then placed in a beaker with enough  $m/1000$  acetate buffer to reach the edge of the crucible (about 50 cc.). After several

hours the weight of the gelatin became about 80 per cent and next day it reached the value of 77 per cent, which then became constant. Its dry weight was then determined and was found to check with the weight as calculated from the loss of water. The amount of water lost here by the 4 per cent gel through "syneresis" was identical with the amount of water lost by a solid block of 4 per cent gel weighing 1.5 gm. and immersed in 150 cc. of liquid with several changes of the outside solution. In this last case it took about 10 days to reach the equilibrium state. In another experiment two solid blocks of 4 per cent gel each weighing about 3.5 gm. were placed in stoppered Pyrex tubes, to one of which was added about 5 cc. of  $H_2O$  pH 4.7, while the other tube had no water at all. But the pressure of the block of gelatin on the glass was enough to start the diffusion of fluid from the gelatin with the result that the loss in weight of both blocks was identical, each losing 17 per cent in weight after 3 days.

In complete absence of water the process of syneresis is very slow due to the slow rate of diffusion of water through the dry surface of the solid gelatin. A trace of water placed on the surface through actual addition or through pressure is enough to start a rapid diffusion of the water from the solid gelatin. A similar occurrence takes place when a water-permeable collodion bag is filled with water and a moderate pressure applied to it. The rate of diffusion of water from the bag is much greater when the bag is immersed in water than when the bag is placed in a moist chamber after it has been dried carefully with a towel. In an actual experiment the writer found that while it took only about 1 minute for a definite volume of water under a pressure of 10 cm. mercury to diffuse out from a collodion bag when immersed in water, it required about 20 minutes for the same volume of water under the same pressure to diffuse out from the same bag after it was dried outside with a towel and placed in a closed tube *over* water. When the bag was immersed in water again the diffusion became as rapid as before.

#### *The Mechanism of Syneresis.*

The experiments thus far described prove that syneresis of gelatin can be conveniently studied by measuring the loss of water in dilute gelatin blocks when kept in dilute buffer pH 4.7 at low temperature,

since the same quantity of water is lost finally whether the block is immersed in a large volume of water or when syneresis is initiated through mechanical forces such as shaking, pressure, etc. Fig. 2 shows the rate of gaining or losing water by isoelectric gels of various gelatin content when kept in  $M/1000$  acetate buffer pH 4.7 at  $5^{\circ}\text{C}$ . The equilibrium values of the concentrations of gelatin in the various gels were plotted separately in Fig. 3 against the original concentra-

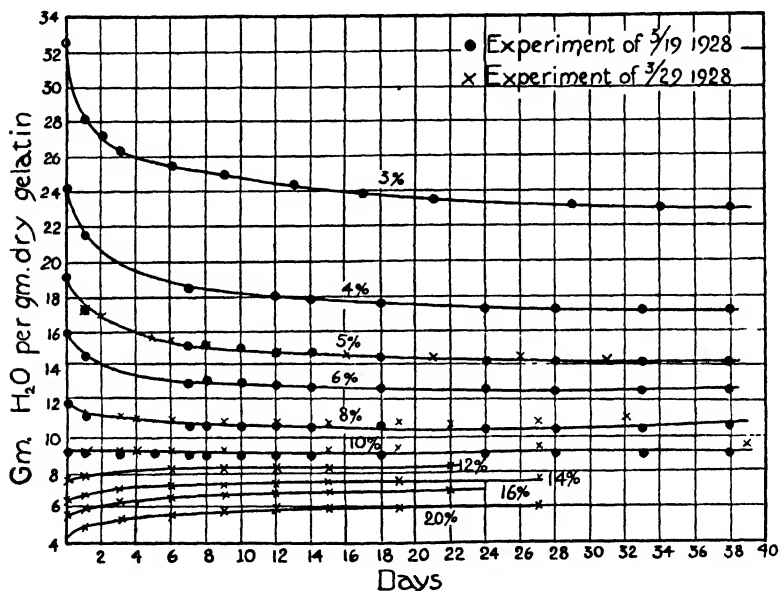


FIG. 2. Swelling of various concentrations of isoelectric gelatin blocks in  $M/1000$  acetate buffer pH 4.7 at  $5^{\circ}\text{C}$ . The gelatin was allowed to set for 24 hours at  $5^{\circ}\text{C}$ . before placing it in the buffer solution.

tions. It is seen that all the points lie on a smooth curve, no matter whether there has been positive or negative swelling, thus suggesting that there is a common mechanism to both forms of swelling. The theoretical relation between the original and final concentrations of a swelling gel has been developed by Northrop<sup>8</sup> for concentrations of

<sup>8</sup> Northrop, <sup>6</sup> p. 898.

gelatin above 10 per cent. His theory is based on the assumption that a block of gelatin behaves like a perfect elastic body, namely that in accordance with Hooke's law any strain in the block is proportional to the stress producing it. A block of freshly set gel is under no strain

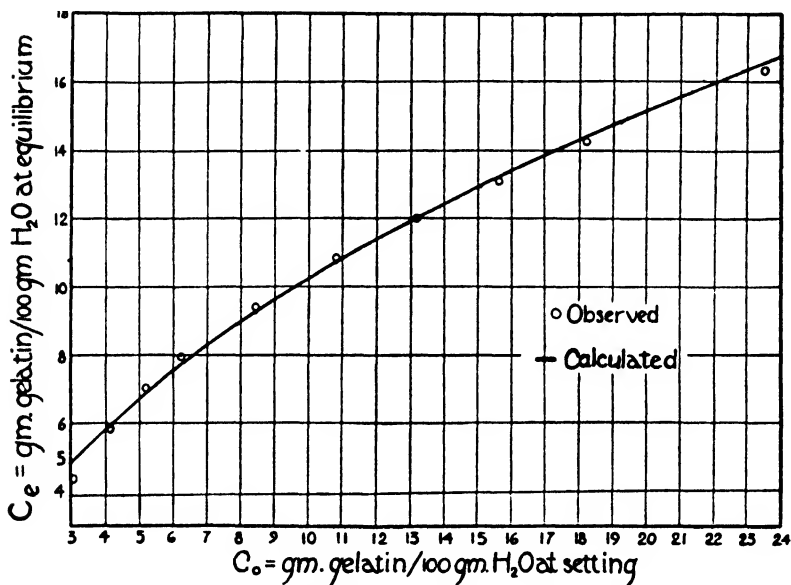


FIG. 3. Relation between the original and the final concentrations of the gelatin in swelling of gels. The dots are the experimental values. The smooth curve represents the theoretical relation:

$$C_e = -\frac{K_e - 140}{26.6} + \sqrt{\left(\frac{K_e - 140}{26.6}\right)^2 + \frac{K_e}{13.3} C_o}$$

where  $K_e = 195$ .

but as soon as it is placed in water the block swells under the influence of a certain swelling pressure  $P$  and a strain is produced in the block. At equilibrium we have

$$P = K_e \frac{V_e - V_o}{V_o} \quad (1)$$

Where  $K_s$  is bulk modulus of elasticity of the block and  $V_o$  and  $V_s$  are cubic centimeters of water per gram of dry gelatin at setting and at equilibrium respectively.

The swelling pressures of gels of higher than 10 per cent have been

TABLE IV.

*Application of Northrop's Formulas for Swelling of Gels.*

$$\frac{1330}{V_s} - 140 = K_s \frac{V_s - V_o}{V_o} \text{ and } P = \frac{1330}{V_s} - 140$$

$K_s$  = Bulk modulus of elasticity in mm. Hg of pressure.

$V_o$  = cc. H<sub>2</sub>O per gm. gelatin at setting.

$V_s$  = cc. H<sub>2</sub>O per gm. gelatin at equilibrium.

$P$  = Swelling pressure in mm. Hg.

Concentration at setting of gelatin in gm per 100 cc solution	$V_o$	$V_s$	$P$	$K_s = \frac{P \times V_o}{V_s - V_o}$	$C_o = \frac{100}{V_o}$ gm gel per 100 cc H <sub>2</sub> O	$C_s = -\frac{K_s - 140}{26.6} + \sqrt{\left(\frac{K_s - 140}{26.6}\right)^2 + \frac{K_s}{13.3} C_o}$	$C_s$ observed $= \frac{100}{V}$
3 0	32 5	23 0	-82 2	281	3 07	4 95	4 35
4 0	24 2	17 0	-61 8	208	4 13	5 98	5 88
5 0	19 2	14 2	-47 0	180	5 18	6 90	7 04
6 0	16 0	12 6	-34 5	163	6 25	7 72	7 94
8 0	11 80	10 65	-16 0	164	8 47	9 26	9 40
10 0	9 25	9 20	+4 5		10 80	10.70	10 86
12 0	7 60	8 35	+19 5	198	13 15	11.98	11 98
14 0	6 42	7 60	+35 0	191	15 6	13 20	13 15
16 0	5 50	7 00	+50 0	183	18 2	14 40	14 30
20 0	4 26	6 10	+78 0	181	23 5	16 60	16 40
Average. ....				195			

measured directly and found that they can be expressed empirically as the following function of  $V_s$ , namely,

$$P = \frac{1330}{V_s} - 140 \quad (2)$$

Combining the two equations we get the following relation between  $V_o$  and  $V_s$ :

$$\frac{1330}{V_s} - 140 = K_s \frac{V_s - V_o}{V_o} \quad (3)$$

As shown by Northrop, this relation holds well for the swelling of blocks of gels of a gelatin content of more than 10 gm. per 100 cc.  $H_2O$ . The same formula was applied by the writer to the cases of negative swelling of gels of lower than 10 per cent with the following results.

Table IV gives the values of  $K_s$  as calculated from the known values of  $V_s$  and  $V_o$ . It is seen that  $K_s$  is practically constant, varying as much in the cases of negative swelling as in the cases of positive swelling. Equation (3) can be easily used to find the values of the equilibrium concentrations of the gelatin in the gels in grams per 100 cc.  $H_2O$  for the various concentrations used, namely,

$$C_s = -\frac{K_s - 140}{26.6} + \sqrt{\left(\frac{K_s - 140}{26.6}\right)^2 + \frac{K_s}{13.3} C_o}$$

where

$$C_o = \frac{100}{V_s} \text{ and } C_s = \frac{100}{V_o}$$

The calculated values of  $C_s$  as well as the observed ones are given in the last two columns of Table IV. They are practically identical with the exception of the lowest concentration. This is also shown clearly on Fig. 3 where the calculated values of  $C_s$  are plotted on the smooth curve.

Thus it is evident that the same laws which hold for swelling of gels of a gelatin content higher than 10 gm. per 100 cc.  $H_2O$  hold also for the process of giving off water by gels of a lower gelatin content. The active force  $P$  in both processes can be calculated by means of equation (1). The values for  $P$  are positive in the high concentrations and negative in the lower concentrations, all lying on one straight line which crosses the zero axis at a concentration of gelatin of about 10 per cent (see Fig. 4).

#### *What is Swelling Pressure?*

It has been assumed by Northrop and the writer<sup>9</sup> that the active force  $P$ , *i.e.*, the swelling pressure in gels, is brought about by the

<sup>9</sup> Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 162.

osmotic pressure of the soluble ingredient in the gel, and is numerically equal to it. But the fact that the swelling pressure becomes negative in gels of a gelatin content of less than 10 per cent indicates that osmotic pressure is not the only factor which causes swelling, that there is also another force which evidently works in the opposite

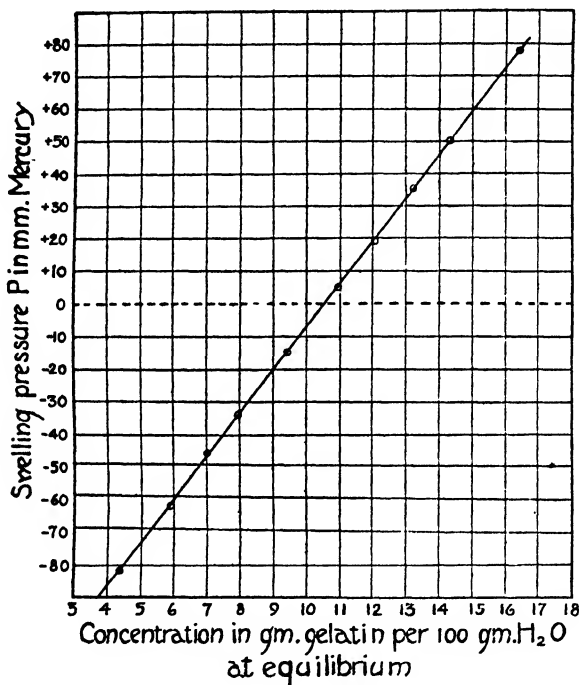


FIG. 4. Swelling pressure of gels of various gelatin content pH 4.7 at 5°C., from the relation  $P = 13.3 C_s - 140$ .

direction to that of the osmotic force, and that the observed swelling pressure is the resultant of the two oppositely acting forces. It will be shown in the following discussion that the force that causes shrinking of dilute gels is connected with the micellar structure of gelatin solutions and is brought about by the strain prevalent in the micellæ before the gelatin has set to a gel.



Loeb<sup>10</sup> first established the theory that the increase of hydration of gelatin in solution due to a change in pH, as shown by viscosity measurements, is brought about by the swelling of the micellæ in the gelatin solution. The micellæ swell because of a higher osmotic pressure inside of the micellæ than outside due to a difference in the ion distribution in accordance with the law of "Donnan equilibrium." The writer<sup>11</sup> has further developed Loeb's idea of hydration by osmosis by applying it to the case of hydration of gelatin at its isoelectric point, where in the absence of diffusible ions no Donnan equilibrium occurs. It was, namely, shown that the hydration of isoelectric gelatin in solution, as demonstrated by viscosity and osmotic pressure measurements, is caused by the fact that each micella in gelatin consists of an insoluble shell containing a definite amount of a soluble ingredient of gelatin. The latter exerts an osmotic pressure on the micella and brings about an inflow of water into it until the osmotic pressure in the micella is balanced by the total osmotic pressure of the solution acting against it and its elastic pressure. The equilibrium state can be expressed as  $P_i - P_o = Eq$  where  $P_i$  and  $P_o$  are the osmotic pressures inside and outside of the micella, respectively,  $E$  is a constant proportional to the bulk modulus of elasticity of the micella, and  $q$  is the amount of water of hydration per gram of gelatin. At low concentration of gelatin the outside osmotic pressure is small, hence the micellæ take up individually relatively large amounts of water. But as the total concentration increases the opposing outside osmotic pressure increases and the micellæ swell less, with the result that  $q$ , *i.e.* the amount of hydration per gram of gelatin gradually becomes less and less. Thus, although the micellæ are at equilibrium with the outside solution, they are still under a strain exerted by a pressure equal to  $Eq$ , the magnitude of which decreases with the increase in the total concentration of gelatin.

*The Theory of the Rôle of the Micellæ in Swelling of Gelatin.*

The hydrated micellæ in a gelatin solution are kinetically free and are able to exert osmotic pressure as any other particles or molecules.

<sup>10</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 2nd edition, 1924, 270.

<sup>11</sup> Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 811.

The first effect of cooling of a gelatin solution is a decrease in the kinetic energy of the micellæ. Their motion becomes sluggish with more chance to adhere to each other, until they begin to form fibrils. Gradually the fibrils interlace and form a network occluding the rest of the gelatin solution. In the absence of electrolytes most of the soluble gelatin becomes insoluble or precipitates out as soon as the temperature has fallen low enough to make the solution supersaturated with respect to it. The micellæ which were under a strain before the gelatin began to set, due to the osmotic pressure of the soluble ingredient inside of the micellæ, now shrink and lose water owing to the precipitation of the soluble gelatin inside of the micellæ. This loss of water probably takes place even before the gelatin becomes solid, but as soon as it solidifies the shrinkage of the micellæ becomes slower because of the resistance to the diffusion of the liberated water offered by the dry surface of the gel, as mentioned before. But as soon as the gel is brought in contact with water a rapid diffusion of water from the block of gel takes place. Each individual micella shrinks, with the result that the whole network contracts and it expels not only the water that was inside of the micellæ but also a great deal of water that was held by capillarity between the micellæ. The shrinkage of the micellæ continues until it is balanced by the elastic resistance of the block as a whole.

### *Effect of Concentration of Gelatin.*

As stated before, the strain due to the inner osmotic pressure in each individual micella in a gelatin solution becomes less as the total concentration of the gelatin increases. Hence when the stress on the micella is removed by the "precipitation" of the soluble gelatin there is less contraction in the micellar network of concentrated gels than in the dilute ones, and less syneresis. There is also another factor which makes the syneresis decrease with the increase in the concentration of the gel, namely the osmotic pressure of the gel as a whole. It was shown by Northrop and the writer<sup>12</sup> that even at a temperature as low as 0°C. there is still enough soluble material left in a solid gel to produce osmotic pressure. The amount of soluble material naturally

<sup>12</sup> Northrop and Kunitz,<sup>9</sup> p. 174.

increases with the increase in total concentration of gelatin. Hence concentrated gels not only do not lose water but actually gain water. Thus syneresis and swelling are caused by two opposite forces acting against the elasticity of the solid block of gel, namely the elastic strain in the micellæ and the osmotic pressure of the block as a whole. At low concentration the first one is prevalent, at higher concentrations the second force becomes more important; while at a concentration of

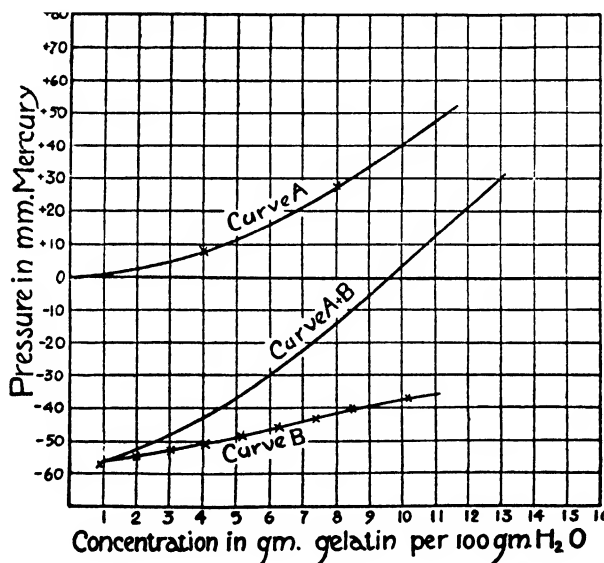


FIG. 5. Curves: *A* for osmotic pressure of isoelectric gelatin solutions at 25°C., and *B* for the stress on the micellæ at 35°C. The resultant curve *A + B* represents the hypothetical curve for swelling pressure of gels.

about 10 per cent the two forces balance each other and the block of gel neither swells nor loses water. The observed swelling pressure  $P$  is then the algebraic sum of the two forces and it is balanced by the elastic pressure of the block of gel.

There seems to be no way to determine directly either the osmotic pressure of a gel or the elastic strain in the micellæ of a gelatin solution at its setting point. Still, an idea of the character of the curve

representing the sum of the two pressures at various concentrations of gelatin may be obtained by plotting the observed values for osmotic pressure of various concentrations of gelatin solutions at a temperature above the setting point, and also the values for the elastic strain in the micellæ as obtained from measurements of viscosity of gelatin solutions. Fig. 5 shows the curve for osmotic pressure at 25°C.<sup>13</sup> plotted as positive, and the curve for  $Eg$  (strain in the micellæ) at 35°C.<sup>14</sup> as

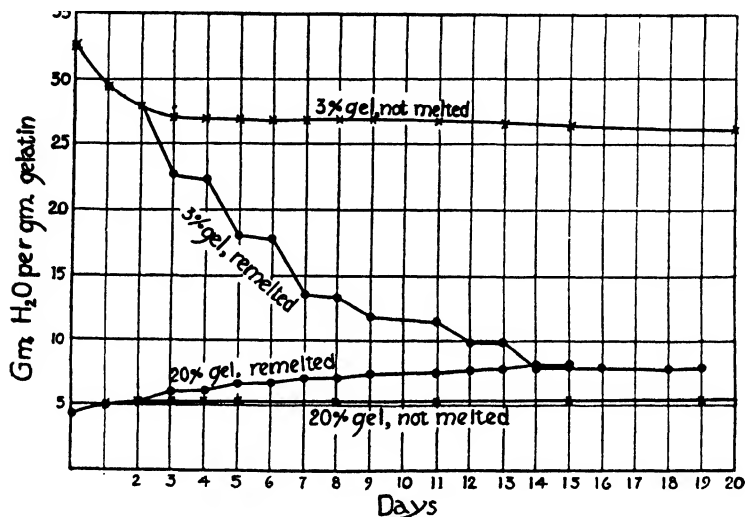


FIG. 6. Effect of reheating of gels on the absorption or liberation of water at 5°C.

negative values of  $P$ . The third curve represents the algebraic sum of the two pressures. It resembles closely the actual curve for swelling pressure as shown in Fig. 4.

#### *Effect of Reheating of Gels.*

The limiting factor in swelling or in shrinking of gels is the elasticity of the block. When the block of gel is heated the elastic strain caused by the swelling is released. Hence when the gel is cooled again and

<sup>13</sup> Northrop and Kunitz,<sup>9</sup> p. 166.

<sup>14</sup> Kunitz,<sup>11</sup> p. 820.

put back into water the gel continues swelling until the swelling pressure is balanced by the new elastic pressure. This has been shown by Northrop<sup>15</sup> to hold true for positive swelling. The writer tried the effect of reheating on two gels, one of which was of 3 per cent gelatin content and the other of 20 per cent content. For this purpose the gelatin solutions were coated on slides, allowed to set for 24 hours, weighed and put into M/1000 acetate buffer pH 4.7. Every 3rd day the gels were heated carefully over a small alcohol flame until melted. They were then allowed to set for 30 minutes, weighed and put back into the solution. The results are given in Fig. 6. The smooth curves are those of blocks which have not been reheated, while the broken curves are those of the reheated blocks. It is to be noticed that the 3 per cent gel keeps on losing water while the 20 per cent continues gaining water until the concentration reaches about 12 per cent in both cases, where no further change takes place on reheating.

*Effect of pH of the Gelatin on the Loss of Water by Gels.*

When blocks of isoelectric gelatin are placed in acid or alkali solution they swell enormously due to the setting up of a Donnan equilibrium. This takes place even with very dilute gels. The same thing happens when the gels are made up of gelatin solutions containing acid or alkali. When put into water or acid or alkali the gel swells. It has also been observed by Jordis<sup>16</sup> and noticed by the writer that gels containing electrolytes are quite stable even in dilute solution, and no syneresis occurs. Quite different results are obtained when the acid or alkali is removed by dialysis.

*Experiment.*- A series of solutions of 3 per cent gelatin were made containing various amounts of HCl or NaOH. The solutions were heated to 50°C., then poured into tubes (2 cc. in each) and allowed to set in a refrigerating room for 24 hours. The blocks were afterwards removed from the tubes, weighed and put into 150 cc. M/30 acetate buffer pH 4.7 where they were kept for several days until the weight became constant. The gels were then transferred to M/1000 acetate buffer pH 4.7 and kept there until new equilibrium had been

<sup>15</sup> Northrop.<sup>6</sup>

<sup>16</sup> Jordis, E., *Z. Electrochem.*, 1902, viii, 677.

established. The M/1000 buffer was renewed several times during the experiment.

The results are given in Fig. 7. It is seen that gels which had a pH other than that of the isoelectric point of gelatin lose more water than gels made of isoelectric gelatin. This shrinking takes place only after the acid or alkali has been removed by neutralization and dialysis. The greater shrinking of gels which contained at setting some acid or

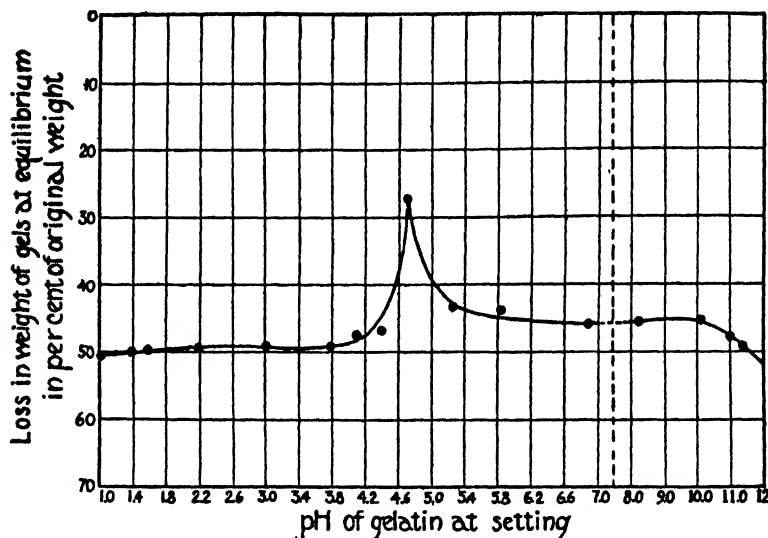


FIG. 7. Effect of pH on syneresis of 3 per cent gel at 5°C.

alkali in solution over those which were electrolyte-free takes place only in gels of low gelatin content. The difference disappears in case of gels of higher concentrations. This is shown in Table V. At a concentration above 6 per cent the loss in water is identical for all gels no matter whether they originally contained acid or alkali or were made up of pure isoelectric gelatin. The peculiar effect of the pH of the original solutions on the loss of water by the gels after the gel was brought back to the isoelectric point becomes clear in view of the theory developed here.

*The Theory of Setting of Gels Containing Acid or Alkali.*

In dilute solutions of gelatin containing moderate amounts of acid or alkali the micellæ are much more swollen than at the isoelectric point due to a greater concentration of diffusible ions inside than outside of the micellæ. Cooling and setting does not change this unequal distribution of ions. Hence, until the ions are removed by dialysis the micellæ do not lose water and no syneresis takes place. But as soon as the acid or alkali is removed by neutralization and dialysis the micellæ shrink much more than in the case of originally isoelectric

TABLE V.

*Swelling of Various Concentrations of Gelatin of Various pH in M/30 Acetate Buffer pH 4.7 at 5°C.*

Expressed as per cent of original weight.

Concentration of gelatin in gm. per 100 cc. solution.....			3	4	5	6	8	10
1928								
2/15	pH 9 0.	15 days in M/30 acetate	75	84		99	110	118
2/24	" 4.7.	15 " " " "	95	96		103	108	114
3/10	" 4.7.	2 " " " "	97	98	100	101	106	110
3/10	" 2.0.	2 " " " "	83	90	95	101	106	111
3/10	" 4.7	2 " " " " then 8 days in H <sub>2</sub> O pH 4.7	79	85	87	88	95	102
3/10	" 2.0	2 days in M/30 acetate, then 8 days in H <sub>2</sub> O pH 4.7	57	70	77	85	93.3	101

gels. With increase in the concentration of gelatin the pH effect on the swelling of the micellæ in a gelatin solution diminishes rapidly, as shown by viscosity measurements,<sup>17</sup> with the result that the pH effect on syneresis in the solid gels is also diminished with increase in concentration of the gelatin. There is also another factor in the effect of acid or alkali on syneresis, namely the solubility effect which is shown also by salts.

*The Effect of Salts on Syneresis.*

It is generally known that salts affect the solubility of gelatin and the rate of setting (Levites<sup>18</sup>). At temperatures above the setting point of

<sup>17</sup> Kunitz,<sup>11</sup> p. 832.

<sup>18</sup> Levites, S. J., *Z. Chem. u. Ind. Kolloid.*, 1907, ii, 161.

solutions of gelatin salts split the micellæ into smaller units thus giving rise to higher osmotic pressures (Northrop and Kunitz<sup>19</sup>). There is very little action of the salts on the soluble fraction of gelatin, which is in solution even in the absence of salts. The total volume of the micellæ is not changed to any considerable extent, and the viscosity of the gelatin solutions is only slightly increased by addition of salt. As a salt-free dilute solution of isoelectric gelatin is allowed to set the soluble fraction both inside and outside of the micellæ gradually

TABLE VI.

*Effect of NaCl on Swelling of 3 Per Cent Gelatin Gels.*

Measured after 24 hours in solution.

		Weight in per cent of original weight of block.						
Concentration of NaCl pH 4.7 .....		0 (H <sub>2</sub> O)	M/256	M/128	M/64	M/32	M/16	M/8
I.	3 per cent salt-free isoelectric gelatin blocks put into salt solutions	88.6	94.0	95.4		100	102	104
II.	3 per cent isoelectric gelatin blocks made up in varying concentrations of NaCl and put into the corresponding salt solutions	86.6	88.5	87.4	89.4	91.2	93.2	94.6
III.	3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl and put into H <sub>2</sub> O pH 4.7	90.0	86.0	84.0	82.0	80.0	77.0	73.0
IV.	3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl. Kept in M/1000 acetate buffer pH 4.7 until equilibrium was established, and then weighed	71.0		60.0	56.0	55.0	54.0	50.0

“precipitates” out during the setting. As stated before, the micellæ lose some of their water of hydration during setting, and the block of gel begins to lose weight almost immediately after it sets if the conditions for diffusion of the water are favorable.

In the presence of salts the setting is slower, first because of the finer state of the micellæ, and secondly because the salts prevent the

<sup>19</sup> Northrop and Kunitz,<sup>7</sup> p. 332.



precipitation of the "soluble" ingredient of gelatin, as shown by higher swelling of isoelectric gelatin in the presence of salts. The micellæ lose much less water during setting than in the absence of salts. It is possible that the micellæ even take up water during setting in the presence of salts because of the reduction of the outside osmotic pressure due to the immobilization of the micellæ.

When the salt-containing gel is put in  $H_2O$  pH 4.7 or dilute buffer of the same pH, the salt dialyzes out and the soluble gelatin both inside and outside precipitates out as in case of originally salt-free gels. The stress on the micellæ due to the greater concentration of soluble

TABLE VII.

*Effect of M/8 NaCl on Swelling of Concentrated Gels of Isoelectric Gelatin at 5°C.*

Gels were made up in M/8 NaCl, allowed to set for 24 hrs and then put into M/1000 acetate buffer pH 4.7. Buffer changed several times.

Concentration of gelatin in gm per 100 cc solution	10	12	14	16	20
Cc $H_2O$ per gm of gelatin at setting	9 20	7 55	6 37	5 48	4 23
" " " " " " " " equilibrium.	9 20	8 30	7 50	6 90	6 00
Per cent increase	0	+10 0	+17 6	+26 0	+42 0

Same gels but without salt					
Cc $H_2O$ per gm gelatin at setting	9 25	7 60	6 42	5 50	4 26
" " " " " " " " equilibrium	9 38	8 35	7 60	7 00	6 10
Per cent increase	+1 5	+10 0	+18 5	+27 5	+43 0

gelatin inside than outside is removed and the micellæ shrink, thus bringing about a greater loss of water due to syneresis in salt-containing gels than in salt-free gels of pH 4.7, as shown in Table VI. It is interesting to observe that the salt-containing gel, which unlike the salt-free gel is not turbid in appearance, does not assume any turbidity even after the salt is dialyzed out. On the other hand when a milky white salt-free gel is placed in salt solution or acid the gel swells but the turbidity persists, thus proving the turbidity is caused by the structure of the micellæ before setting. The turbidity is apparently caused only by coarse micellæ. The splitting of micellæ into finer elements by salts prevents turbidity formation.

*Is the Effect of Salt on Syneresis Due to a Change in the Bulk Modulus of Elasticity of the Block?*

A possible explanation of the peculiar effect of dilute salt solutions on syneresis is that the elasticity of the gel is affected by the presence of salt in it and that even after removal of the salt from the gelatin by dialysis the elasticity of the block still remains altered. The change expected would be a decrease in the bulk modulus so that a smaller

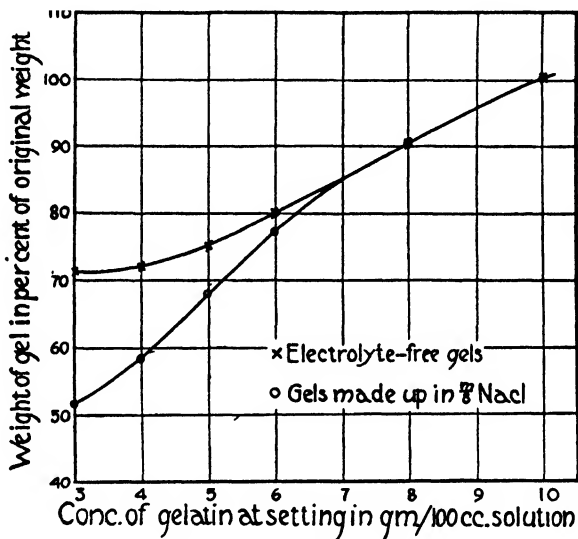


FIG. 8. Effect of salt on syneresis of various concentrations of gelatin blocks in  $M/1000$  acetate buffer pH 4.7 at  $5^{\circ}\text{C}$ .

force is required to compress the block. But if salts in low concentrations affect the elasticity of a gel then it is to be expected in gels of high gelatin content, that those which originally contained salt and from which the salt was afterwards removed would swell more than gels made up of ordinary isoelectric gelatin. Table VII shows that this is not the case. The swelling of gels of a gelatin content of more than 10 per cent is identical whether the gels originally contained salt or not. The effect of salt is only on the shrinking of gels of low concen-

trations, and it disappears at a concentration of about 8 per cent, as shown in Fig. 8, and is best explainable by the effect of salts on the solubility of the gelatin in the micellæ.

*The Solubility Effect of Dilute Acid or Alkali on Syneresis.*

Acids and alkalies in addition to their pH effect on gelatin have also an enormous effect on the solubility of gelatin. This explains why the curve for syneresis effect of pH does not give a maximum point similar to the pH viscosity curves. A continuous increase in acid or alkali concentration has the same effect on the solubility of gelatin as addition of large amounts of salt and at higher concentrations of acid or alkali the solubility effect prevails.

SUMMARY.

1. When solid blocks of isoelectric gelatin are placed in cold distilled water or dilute buffer of pH 4.7, only those of a gelatin content of more than 10 per cent swell, while those of a lower gelatin content not only do not swell but actually lose water.

2. The final quantity of water lost by blocks of dilute gelatin is the same whether the block is immersed in a large volume of water or whether syneresis has been initiated in the gel through mechanical forces such as shaking, pressure, etc., even in the absence of any outside liquid, thus showing that syneresis is identical with the process of negative swelling of dilute gels when placed in cold water, and may be used as a convenient term for it.

3. Acid- or alkali-containing gels give rise to greater syneresis than isoelectric gels, after the acid or alkali has been removed by dialysis.

4. Salt-containing gels show greater syneresis than salt-free gels of the same pH, after the salt has been washed away.

5. The acid and alkali and also the salt effect on syneresis of gels disappears at a gelatin concentration above 8 per cent.

6. The striking similarity in the behavior of gels with respect to syneresis and of gelatin solutions with respect to viscosity suggests the probability that both are due to the same mechanism, namely the mechanism of hydration of the micellæ in gelatin by means of osmosis as brought about either by diffusible ions, as in the presence of acid or

alkali, or by the soluble gelatin present in the micellæ. The greater the pressures that caused swelling of the micellæ while the gelatin was in the sol state, the greater is the loss of water from the gels when the pressures are removed.

7. A quantitative study of the loss of water by dilute gels of various gelatin content shows that the same laws which have been found by Northrop to hold for the swelling of gels of high concentrations apply also to the process of losing water by dilute gels, *i.e.* to the process of syneresis. The general behavior is well represented by the equations:

$$P_1 - P_2 = K_s \frac{V_s - V_e}{V_e}$$

and

$$P_1 - P_2 = \frac{1330}{V_e} - 140$$

where  $P_1$  = osmotic pressure of the soluble gelatin in the gel,  $P_2$  = stress on the micellæ in the gelatin solution before setting,  $K_s$  = bulk modulus of elasticity,  $V_s$  = volume of water per gram of dry gelatin at setting and  $V_e$  = volume of water per gram of gelatin at equilibrium.

The writer wishes to express his indebtedness to Dr. John H. Northrop for very valuable suggestions and advice.

## FRACTIONATION OF GELATIN.

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It has been shown by the writers<sup>1</sup> that such phenomena as the swelling of electrolyte-free gelatin blocks, the high viscosity of gelatin solutions, the decrease of the pH effect on viscosity with increase in the concentration of gelatin solutions, and, finally, the peculiar phenomenon of shrinking of dilute gels when placed in cold water, are explainable on the assumption that gelatin consists of a mixture of at least two substances, one of which is soluble in cold water, while the other is insoluble even in warm water. Actually, it has been found possible to isolate from gelatin by means of alcohol precipitation two fractions which differ enormously in their physical properties.<sup>2</sup> One of the fractions has properties similar to those of albumin; it is soluble even in ice water, does not set to a gel, has a low viscosity, and does not give rise to the pH-viscosity curve characteristic for gelatin. Besides, it has an osmotic pressure at its isoelectric point (pH 4.7) considerably higher than that of isoelectric gelatin, from which it has been isolated. The second fraction has properties opposite to those of the first one; it is insoluble in cold water, sets to a gel at a low concentration, swells much less, and has a higher viscosity and lower osmotic pressure than ordinary gelatin. But this fraction is still "soluble" in warm water.

It had been previously found by Schryver<sup>3</sup> and his associates that a soluble material could be obtained from gelatin by allowing isoelectric salt-free gelatin to stand at 14-16°C. Under these conditions the

<sup>1</sup> (a) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 161. (b) Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 811. (c) Northrop, J. H., *J. Gen. Physiol.*, 1926-27, x, 893. (d) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 905. (e) Kunitz, M., *J. Gen. Physiol.*, 1928-29, xii, 289.

<sup>2</sup> Northrop and Kunitz,<sup>1a</sup> p. 167.

<sup>3</sup> Knaggs, J., Manning, A. B., and Schryver, S. B., *Biochem. J.*, 1923, xvii, 473.

gelatin separates out as a clot leaving some soluble protein material in the supernatant fluid.<sup>4</sup> If the precipitate is then suspended in water a lower concentration of the soluble material is found in the supernatant solution. This solution moreover did not have the properties of ordinary gelatin, so that, as these workers point out, it can not be considered as a saturated solution of gelatin. These experiments were repeated and confirmed by the present writers, but it was found that if the gelatin were allowed to stand at 23° instead of 15° a very much larger amount of the soluble material was obtained and this fact was used as the basis of the method of separation used in the present paper.

### *Experimental Procedure.*

A stock of isoelectric gelatin is prepared from Cooper's non-bleached gelatin by the modified Loeb's method as described elsewhere.<sup>5</sup> A solution of 60 gm. air-dried isoelectric gelatin (85 per cent dry weight) is made in 3000 cc. of distilled water, which has been adjusted to pH 4.7 by means of acetic acid, filtered through cotton wool into Pyrex flasks, and put into a water bath kept at a constant temperature of 23°C. A layer of toluene is added to the gelatin to prevent mould and bacterial growth. The gelatin solution gradually turns milky white, thickens, and after several days the thick white gel begins to break up into a gelatinous precipitate which settles slowly. The precipitation may be accelerated by stirring up the gel occasionally. After 5 or 6 days the flasks are removed from the bath, the supernatant solution is decanted as much as possible, and the rest is centrifuged, care being taken not to heat the gelatin in the centrifuge. The centrifuged precipitate is stirred up once or twice in the centrifuge bottles with cold water of pH 4.7 and recentrifuged. It is then melted and made up to about 80 per cent of the original volume of the 2 per cent solution with water of pH 4.7, and allowed to set at 23°C. for the second precipitation. The process is repeated 12 to 15 times until a precipitate is left which does not dissolve even at 50°C. Care has to be taken during the whole process that the pH should remain at 4.7. The final precipitate is washed several times in the centrifuge bottles with hot water, and transferred into 95 per cent alcohol, where it is allowed to remain overnight. The alcohol is then decanted off and the gelatin is ground up in a mortar with ether, which is allowed finally to volatilize.

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<sup>4</sup> Schryver and Thimann<sup>5</sup> were also able to obtain a soluble and insoluble fraction by means of electrolysis of gelatin solutions.

<sup>5</sup> Schryver, S. B., and Thimann, K. V., *Biochem. J.*, 1927, xxi, 1284.

<sup>6</sup> Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927-28, xi, 477. Gelatin prepared in this way is practically free from diffusible nitrogenous material, cf. Knaggs, Manning, and Schryver.<sup>3</sup>

The air-dried precipitate is a fine powder of a light chocolate appearance. The yield is about 1 per cent of the original amount of gela-

TABLE I.  
*Precipitation of Isoelectric Gelatin at 23°C.*

	Volume	Dry gelatin	Strength of solution
	cc	gm	per cent
Original	3000	51	1 66
1st precipitate	2100	43 5	2 07
1st supernatant solution	900	6 9	0 77

TABLE II.  
*Influence of Initial Concentration of Gelatin Solution on Concentration of Gelatin in the Supernatant Liquid after 1st Precipitation at 23°C*

Initial concentration in gm dry gelatin per 100 gm solution	0 6	1 17	1 70	2 35	2 93
Gm dry gelatin per 100 gm supernatant liquid	0 19	0 53	0 68	1 21	1 19

TABLE III  
*Gelatin Found in Supernatant Liquid on Repeated Precipitation of a Gelatin Solution at 23°C Original Amount of Gelatin 51 Gm by Dry Weight*

Precipitation	1	2	3	4	5	6	7	8
Concentration of gelatin in supernatant liquid in gm per 100 cc solution	0 77	0 55	0 48	0 50	0 50	0 40	0 37	0 42
Gm gelatin in the supernatant liquid	6 9	9 5	5 6	1 2	3 1	4 7	2 3	1 9
Precipitation	9	10	11	12	13	14	15	
Concentration of gelatin in supernatant liquid in gm per 100 cc solution	0 31	0 31	0 24	0 15	0 15	0 15	0 12	
Gm gelatin in the supernatant liquid	1 9	2 0	1 5	0 9	0 98	0 61	0 54	

tin The supernatant solutions may be used for preparation of the soluble fraction by means of alcohol, as described elsewhere.<sup>2</sup> If the precipitation is carried out at a lower temperature than 20°C. practically

all of the gelatin comes out as a precipitate with only a trace remaining in the supernatant solution. On the other hand, when the precipitation is done at 23°C. 14 to 18 per cent of the original gelatin remains in the supernatant solution. The distribution of gelatin between the precipitate and the supernatant solution after the first precipitation at 23°C. is shown in Table I.

The concentration of soluble material increases with the total concentration of gelatin, as shown in Table II.

On further precipitation the amount of gelatin in the supernatant solution gradually decreases, as shown in Table III.

#### *The Physical Properties of the Separated Fractions of Gelatin.*

*Solubility.*— The difference in the solubility of the various fractions of gelatin appears even after the first precipitation. Thus, in one of the experiments a solution of the first precipitate and of the first supernatant solution was completely precipitated by means of alcohol, washed with ether, and dried in the air. Suspensions were then made of 0.1 gm. dry powder of the two fractions in 10 cc. of water each, at 25°C. The fraction from the supernatant solution dissolved immediately in the water producing a clear solution, while the fraction from the first precipitate swelled but did not dissolve unless it was heated. After repeated precipitation the solubility of the precipitate becomes less until finally it does not dissolve even in hot water. The insoluble fraction appears only on repeated fractionation. Simple reheating of gelatin without separation of the precipitate does not produce any visible effect on the gelatin. A 2 per cent solution remained unchanged after heating to 50° and cooling to 23° more than 20 times during a period of 2 months.

#### *Swelling of Powdered Gelatin.*

The final precipitate does not swell when suspended in cold water. Its bulk is the same whether it is suspended in water, alcohol, or toluene. This is shown in Table IV. The bulk was determined by centrifuging suspensions of 0.1 gm. of the dry powder in 10 cc. of various liquids to constant volume of sediment. As a comparison suspensions were also made of ordinary isoelectric gelatin which had been precipi-



tated and dried by means of alcohol and ether in the same way as the fractionated gelatin, and also of the precipitate from the first precipitation.

Thus it is seen that when 0.1 gm. of ordinary gelatin is suspended in water it has a bulk of 0.9 cc. when centrifuged as compared with the bulk of 0.25–0.30 cc. when it is suspended in absolute alcohol or toluene; on the other hand, the insoluble fraction of gelatin from the final precipitation has practically the same bulk, 0.35–0.40 cc., no matter whether water, alcohol, or toluene is used. It is to be noted that the insoluble gelatin which had been precipitated only once gives higher swelling in water than ordinary gelatin, which is partly soluble at 25°C.

TABLE IV.  
*Comparison of Swelling of Various Types of Gelatin at 25°C.*

Liquid	Insoluble gelatin from final precipitation	Insoluble gelatin from first precipitation	Normal isoelectric gelatin
	Cc sediment		
Distilled water	0 40	1 20	0 90
Absolute alcohol	0 35	0 20	0 30
Toluene	0 35	0 25	0 25

*Effect of Acid or Alkali on the Swelling of the Insoluble Fraction of Gelatin.*

Addition of dilute HCl or NaOH to a suspension of the insoluble fraction of gelatin in water brings about a considerable swelling of the particles of gelatin, owing to the unequal distribution of the diffusible ions between the particles and the outside solution in accordance with the Donnan equilibrium. The swelling increases with increase in the amount of acid or alkali until a pH about 3.0 on the acid side or 9.0 on the alkaline is reached when the material begins to dissolve on heating, forming a viscous solution. On bringing the solution back to pH 4.7 the material rapidly precipitates out in a flocculent state.

*Swelling of Gels Made of Mixtures of the Soluble and the Insoluble Fractions of Gelatin.*

The mechanism of swelling of gels of isoelectric gelatin has been studied extensively by the writers.<sup>1</sup> The final conclusions reached may

be summarized as follows. There are three forces which affect the swelling of isoelectric gels:

1. The osmotic force due to the presence of a soluble ingredient of gelatin even at temperatures as low as 5°C. This force causes the block of gel to take up water, and is the significant factor in gels of high gelatin content.

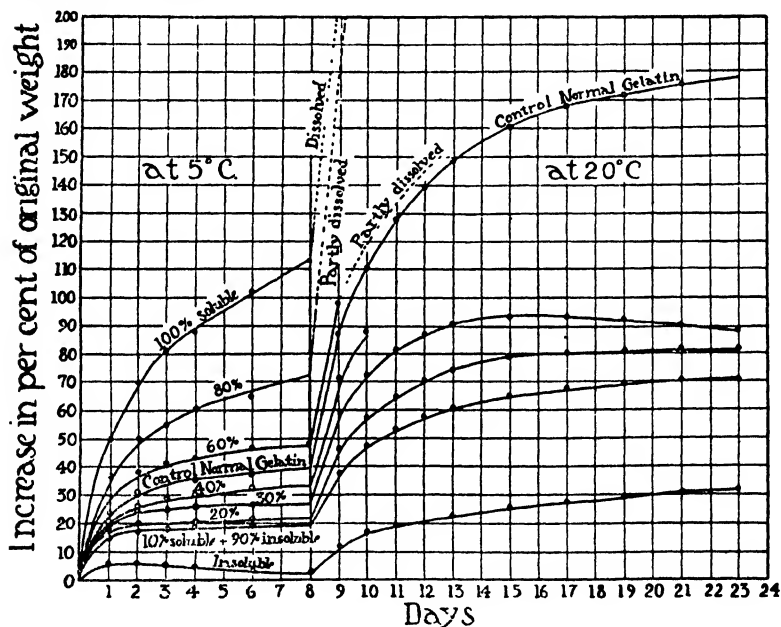


FIG. 1. Swelling of 30 per cent gels consisting of mixtures of insoluble and soluble fractions of gelatin. Blocks of gel weighing about 1.5 gm. each were allowed to swell in 200 cc. M/30 acetate buffer pH 4.7.

2. The residual elastic force in the micellæ, which originates while the gelatin is still in the sol state. This force causes the block of gel to lose water and shrink. It is the significant factor in dilute gels.

3. Finally the elastic force of the block of gel as a whole which resists both the swelling and the shrinking of gels.

According to this viewpoint a concentrated gel made up entirely of the insoluble ingredient should not swell at all in the absence of acid,

alkali, or salt, since there are no soluble molecules to produce an osmotic force. Addition of increasing amounts of the soluble fraction to a gel of the insoluble fraction of gelatin gives rise to osmotic pressures, and increased swelling should be expected. Fig. 1 shows what actually takes place when gels containing various mixtures of the insoluble and soluble ingredients were placed in a  $M/30$  acetate buffer pH 4.7. The experiment was done as follows:

A series of solutions containing 30 gm./100 gm. of solvent was made up of various proportions of soluble and insoluble fractions in warm 0.075  $N$  NaOH. 2 cc. of each solution was poured into a mould of glass tubing, mounted on a paraffin block, and allowed to set for 24 hrs. in the refrigerator at 5°C. The blocks of gel were then removed from the glass tubing, weighed, and put into 200 cc. of  $M/30$  acetate buffer pH 4.7 which had been previously cooled to 5°C. The blocks were weighed at various intervals of time in a refrigerating room which was kept at constant temperature of 5°C. A block of 30 per cent ordinary isoelectric gelatin dissolved in 0.075  $N$  NaOH was prepared and put into 200 cc. of  $M/30$  acetate buffer pH 4.7 as a control. The blocks remained at 5°C. for 8 days, and then were transferred with the outside solutions to a constant temperature water bath of 20°C.

The plotted curves for the rate of swelling of the gels show that at 5°C. the gel consisting of 100 per cent insoluble fraction of gelatin swells slightly if at all, while the addition of the soluble ingredient brings about swelling. The swelling increases rapidly as the proportion of the soluble material increases. Gels consisting of more than equal parts of soluble and insoluble fractions swell more than the normal gelatin control. When transferred to 20°C. all gels begin to swell faster, and those that have been swelling more than the control at 5°C. swell at 20°C. so rapidly that in a day or so they dissolve completely leaving a residue of insoluble gelatin. The gel consisting entirely of the soluble fraction melts immediately when the temperature is raised to 20°C. That the fractionation is not complete yet is shown by the fact that even the 100 per cent *insoluble* gel swells somewhat at 20°C. which indicates that there is still left in it some of the fractions which are soluble at 20°C. It is also possible that the presence of the electrolytes of the buffer affect the solubility of the gelatin as was found before in the case of the swelling of ordinary gelatin. The fractionation of the soluble fraction used in this experiment was purposely stopped at this

point so that a 30 per cent gel at 5°C. would be obtained. By means of further fractionation of the soluble ingredient with alcohol it is very easy to obtain a gelatin fraction which does not set even at 5°C. no matter how concentrated the gel is.

*Negative Swelling of Dilute Gels of Insoluble Gelatin.*

When gels containing less than 10 per cent gelatin are placed in cold water or dilute buffer pH 4.7 the gels not only do not swell but actually shrink and lose water. It has been shown by one of the writers<sup>14</sup> that the force causing the dilute gels to lose water is the elasticity in the micellæ of gelatin. This elastic force, which is brought about by the hydration of the micellæ in the gelatin solution, is due, first, to the presence of soluble gelatin inside of the micellæ and, second, to an excess of diffusible ions inside of the micellæ over the outside solution. The osmotic pressure in the micellæ is therefore higher than in the surrounding solution and water enters until the elastic force is equal to the osmotic pressure. When the gelatin solution sets to a gel the soluble material precipitates and the ions diffuse out when the gel is placed in water. The osmotic pressure is thereby destroyed and the water is forced out by the elasticity of the micellæ, thus bringing about a gradual shrinking and loss of water by the block of gel as a whole. This contraction is greater the greater the amount of water originally held by the micellæ. The amount of water held in them is large in gelatin sols made up in acid or alkali. In ordinary gelatin the micellæ form only a part of the total gelatin, the rest of which is found in solution outside of the micellæ. A solution of the *insoluble* fraction of gelatin in dilute NaOH is not a perfect solution but rather a colloidal suspension of swollen micellæ, as shown by the high viscosity of the mixture. The number of micellæ per gm. of substance then is greater in the case of the insoluble fraction of gelatin than in the case of ordinary gelatin. Hence it is to be expected that when dilute gels are made up of both materials the one consisting of the insoluble component of gelatin should lose more water when put in dilute buffer pH 4.7 than the gel which was made up from ordinary gelatin. That this is actually what happens is shown by the following experiment.

Two solutions were made up each containing 0.1 gm. gelatin + 2 cc.

M/10 NaOH. One was made up of ordinary isoelectric gelatin, while in the other the insoluble fraction was used. Both solutions were allowed to set for 24 hours at 5°C. and then the gels were weighed and each put into 250 cc. M/30 acetate buffer pH 4.7 at 5°C. where they remained for about a month. The weight of the gels was determined at various intervals of time. Fig. 2 shows the results. It is seen that while the block of gel which was made up of ordinary gelatin has lost finally about 22 per cent of its original weight, the one that was made up of the insoluble fraction has lost as much as 68 per cent under the same conditions. The dry weight of the gel was unchanged so that the result is not simply due to solution.

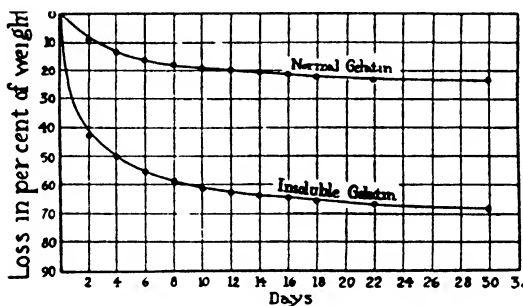


FIG. 2. Negative swelling of 5 per cent gels in M/30 acetate buffer pH 4.7 at 5°C.

### *The Irreversibility of Gelatin Fractionation.*

According to the mechanism of swelling outlined above the soluble ingredient of ordinary gelatin is present both inside and outside of the micellæ. Each micella may be compared to a cell, filled with soluble gelatin; the cell wall, which is impermeable to the soluble gelatin, consists of the insoluble fraction of gelatin. The number of micellæ in a gelatin solution increases with the decrease in temperature until the setting point is reached. When a dilute solution of salt-free isoelectric gelatin is left at a temperature of about 23°C. the micellæ together with the contained soluble fraction of gelatin in them gradually settle down as a gelatinous precipitate, while the supernatant liquid consists of a

solution of practically pure soluble material. The process of removal of the soluble fraction of gelatin from inside of the micellæ is a difficult one owing to the impermeability of the micella wall. Only through a long series of reprecipitations accompanied by repeated heating of the solution is it possible to obtain an insoluble fraction which is apparently free from the soluble component. The liberation of the soluble material is probably due to a rupture of the micellæ by osmotic pressure. In a gelatin solution some soluble material exists both inside and outside of the micellæ. When the gelatin is allowed to set in dilute solution some of the soluble material escapes and is removed. On remelting the gelatin the osmotic pressure difference between the inside and outside of the micellæ is greater than before and they therefore swell more. That this actually happens is shown by the fact already mentioned that a partially purified fraction has a higher viscosity than the original gelatin. As the purification proceeds the swelling increases until the micellæ rupture and liberate the soluble material. The ruptured micellæ thus give rise to the insoluble fraction of gelatin. This fraction is not only insoluble in hot or cold water in the absence of acid or alkali, but it will not mix even with the soluble fraction or with ordinary gelatin to form a homogeneous solution unless it is first dissolved in acid or alkali; on bringing the solution back to pH 4.7, however, the insoluble fraction precipitates out again. In order to resynthesize gelatin from its final fractions it is not enough to mix the fractions in the right proportions but it is necessary to reintroduce the molecules of the soluble material into the insoluble particles; or, in other words, to fill again the micellæ cells with a substance for which the cell wall is impermeable. This may be partially accomplished in the case of a solid block since the soluble material is held in the network of micellæ forming the block but it has not been possible to introduce the soluble material into the individual micellæ. It is probable that in the manufacture of gelatin the soluble material is actually formed inside the micellæ.

It could be assumed that the soluble fraction was formed from the gelatin by an irreversible chemical reaction and hence mixing the fractions would not give the original gelatin. This assumption appears improbable since, as was shown, repeated heating and cooling does not result in the separation of the two fractions. It has also been found by

Alsberg and Griffing<sup>7</sup> that gelatin becomes partly soluble in cold water after prolonged grinding. If the grinding were sufficient to rupture the micellæ, the soluble material would be liberated.

*Isolation of an Insoluble Substance from Gelatin by Partial Acid Hydrolysis.*

A substance similar in many respects to the insoluble ingredient of gelatin described above is obtained when a 5 per cent solution of gelatin is partially hydrolyzed in  $N/10$  HCl at 90°C. for about 2 hours. At this stage the gelatin no longer sets when cooled. On neutralization of the solution a flocculent precipitate appears, which can be centrifuged off and washed with water. On drying with alcohol and ether it gives a light brown powder similar to the one obtained by fractional precipitation. The yield is about 0.2 per cent. The powder is insoluble in hot or cold water, and swells considerably on addition of acid or alkali to its suspension in water until it is finally dissolved, as is the case with the insoluble component of gelatin from fractional precipitation. The only difference between the two substances is their behavior with regard to setting. It has been mentioned before that when an alkaline solution containing 5 per cent or more of the insoluble fraction of gelatin is left to cool to 5°C. it gradually sets to a gel. This does not happen with the insoluble substance obtained by acid hydrolysis of gelatin. A solution of it in dilute NaOH is very viscous, but it does not set even at a concentration of 10 gm. per 100 cc. solution. It is possible that the setting of gelatin depends on the presence of an ingredient which still exists in the material obtained by fractional precipitation but which is destroyed on hydrolysis with acid.

SUMMARY.

1. It is possible to fractionate gelatin by means of reprecipitation at 23°C. of a salt-free solution of pH 4.7 into two fractions, one of which is soluble in water at any temperature, and a second one which does not dissolve in water even when heated to 80°C.
2. The proportion of the soluble fraction in gelatin is much greater than of the insoluble one.
3. The insoluble fraction of gelatin does not swell when mixed with water, but it does swell in the presence of acid and alkali which finally dissolve it.
4. Blocks of concentrated gel made by dissolving various mixtures of

<sup>7</sup> Alsberg, C. L., and Griffing, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1925-26, **xxiii**, 142.

the soluble and insoluble fractions of gelatin in dilute NaOH swell differently when placed in large volumes of dilute buffer solution pH 4.7 at 5°C. The gel consisting of the insoluble material shows only a trace of swelling, while those containing a mixture of soluble and insoluble swell considerably. The swelling increases rapidly as the proportion of the soluble fraction increases.

5. A 5 per cent gel made up by dissolving the insoluble fraction of gelatin in dilute NaOH loses about 70 per cent of its weight when placed in dilute buffer pH 4.7 at 5°C. A similar gel made up of ordinary gelatin loses only about 20 per cent of its weight under the same conditions.

6. It was not found possible to resynthesize isoelectric gelatin from its components.

7. An insoluble substance similar in many respects to the one obtained by reprecipitation of gelatin is produced on partial hydrolysis of gelatin in dilute hydrochloric acid at 90°C.



THE EFFECT OF THE HYDROGEN ION CONCENTRATION  
ON THE RATE OF HYDROLYSIS OF GLYCYL GLY-  
CINE, GLYCYL LEUCINE, GLYCYL ALANINE,  
GLYCYL ASPARAGINE, GLYCYL ASPARTIC  
ACID, AND BIURET BASE BY EREPSIN.

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INTRODUCTION.

The characteristic effect of the hydrogen ion concentration on the activity of erepsin was explained by Euler (1) on the assumption that the enzyme attacked only the ionized form of the substrate. Michaelis (2), on the other hand, has ascribed the effect of the hydrogen ion concentration on the activity of several other enzymes to changes in the ionic species of the enzyme. One of the writers (3) found in the case of pepsin or trypsin that the pH-activity curve varied with different proteins and that the general shape of the curves could be predicted by assuming that pepsin acted upon the positive protein ion and trypsin on the negative protein ion, without making any assumptions in regard to the ionization of the enzyme. The hydrolysis of dipeptides by erepsin furnishes a favorable test of these assumptions since the equilibria governing the substrate are better known than is the case with most enzyme reactions. If the effect of pH depends primarily on changes in the condition of the enzyme, the pH-activity curves for various dipeptides should be the same, while if the pH effect is primarily on the substrate then the pH-activity curves for peptides having different titration curves should be different and should vary with the titration curves of the peptides. The rate of hydrolysis of glycyl glycine (GG), glycyl alanine (GA), glycyl leucine (GL), glycyl asparagine (GApNH<sub>2</sub>), glycyl aspartic acid (GAp) and biuret base (B) at various pH values has been determined under

conditions such that the relative rate of hydrolysis (at the different pH values) was independent (or nearly so) of the enzyme concentration and of the substrate concentration. It was found that the pH-activity curve shifts with the titration curve of the substrate so that the condition of the substrate is evidently one of the determining factors. The equilibrium conditions in the substrate, however, are not sufficient in general to enable the entire pH-activity curve to be calculated and it is necessary to assume in addition that the enzyme is a weak acid or base with a dissociation constant of about  $10^{-7.6}$  (i.e.,  $pK_s' = 7.6$ ). Independent evidence for this assumption has been found in the effect of the pH on the rate of destruction of the enzyme and the effect of neutral salts on the rate of the reaction. If it is then assumed that the reaction takes place between the ionic forms given in Table I the pH-activity curves may be calculated with a fair degree of accuracy.

TABLE I.

*The Ionic Species of Substrate and Enzyme between Which Reaction May Take Place.*

Substrate	I. Low pH form of substrate acted on by high pH form of enzyme		II. High pH form of substrate acted on by low pH form of enzyme	
	Peptides	Biuret base	Peptides	Biuret base
Substrate charge	Zwitterion $\pm$	Cation $+$	Anions — or — Zwitteranion* $\pm -$	Non-ionized 0
Enzyme charge if an acid if a base	Anion (—) Non-ionized (0)		Non-ionized (0) Cation (+)	

\* The dianion (— —) and zwitheranion ( $\pm -$ ) forms refer only to glycyl aspartic acid (of the substances under discussion).

### *Experimental Methods.*

*Preparation of Erepsin Solution.*—Fresh small intestine of swine was thoroughly washed and run through a chopper several times. The resulting thick paste was mixed with an equal volume of glycerin and toluene was added. It was allowed to stand at 17–20°C. for 3 days and then filtered through cheese-cloth. The resulting solution keeps indefinitely at 0°C. Before use the solution was further

purified by dialysis in a strong, tightly stoppered collodion sac against cold running tap water for 24 hours. A heavy flocculent precipitate forms during this time and is filtered off. The filtrate is again dialyzed for 24 hours against cold tap water and again filtered. This filtrate is used in a final dilution of about 1 to 10. In this dilution the formol titration is negligible and the hydrolysis of 0.02 M GG is half completed in about 2 hours. Dry weight determinations on this solution give a few per cent residue which, however, seems to consist largely of glycerin so that the value is of no significance. The solution has no action on gelatin.

*Preparation of the Dipeptides.*—The dipeptides, except biuret base, were prepared from racemic amino acids according to Fischer (4) and were recrystallized until the melting points and amino nitrogen figures were correct.

The biuret base was prepared according to Curtius (5). The substance obtained, however, was insoluble in chloroform but in other respects agreed with that described by Curtius. When dissolved in water it gave a solution of about pH 8.0 and when titrated with acid and alkali a titration curve having a pK value of about 8.3 was obtained. On complete hydrolysis with erepsin the equivalent of 3 carboxyl groups were liberated so that it was assumed that the substance was a mixture of the free base and the carbonate. Lack of material prevented more definite identification.

*Method of Following the Reaction.*—In order to determine the pH-activity curve it is necessary to carry out the reaction at constant pH. The pH may be kept constant by the use of phosphate buffers or by using only the first part of the curve. In the latter case the peptides serve as the buffer if in addition M/100 Na acetate is added to buffer the range from 5.0 to 7.0. Both methods were used at first and it was found with glycyl glycine that the results were the same. The presence of phosphate retards the reaction and as it was later found necessary to work with as dilute erepsin as possible the later experiments were done in the absence of phosphate and the reaction followed only until 20 per cent complete. The pH was measured electrometrically before and after the experiment and was found not to vary sufficiently to affect the results.

*Method of Titration. Phosphate Present.*—Sufficiently large samples were taken, depending on the concentration of the peptide, to give an initial formol titration of 2 to 5 cc. 0.01 M NaOH. 1 cc. 10 per cent ferric chloride solution was added and sufficient 0.2 M NaOH to make the solution alkaline to phenolphthalein. The phosphate, iron and any protein present were thus precipitated and filtered off. The filtrate was brought to pH 5, 1 cc. formalin added and the solution titrated as previously described (6).

In the absence of phosphate the formalin was added and the sample titrated direct. All titrations were made in triplicate and usually agreed within 0.05 cc. 0.01 M NaOH. Six to ten complete series of hydrolysis at intervals of about 0.5 pH from 5 to 9 were run on each peptide.

*Temperature.*—All experiments, except those on the rate of inactivation of the enzyme, were made at 25°C.

*Method of Calculation, etc.*—According to the simple theory of a catalytic reaction the course of the reaction is predicted by the monomolecular formula and the velocity constant obtained in this way should be independent of the substrate concentration and proportional to the catalyst concentration. In the case of enzyme reactions this is not usually the case, the velocity constants decrease as a rule as the substrate concentration increases. If this effect of the substrate concentration on the velocity constant depended on the pH of the solution it is evident that different pH-activity curves would be obtained depending on the concentration of substrate. To be significant the curves must depend only on the pH and be independent of the substrate concentration. It was found in the case of glycyl leucine that the velocity constants decreased much more rapidly with increasing substrate concentration at pH 8.5 than at pH 6.0 so that the relative rate of hydrolysis at pH 8.5 compared to pH 6.0 decreased with increasing glycyl leucine concentration. In order to avoid this difficulty it was necessary to carry

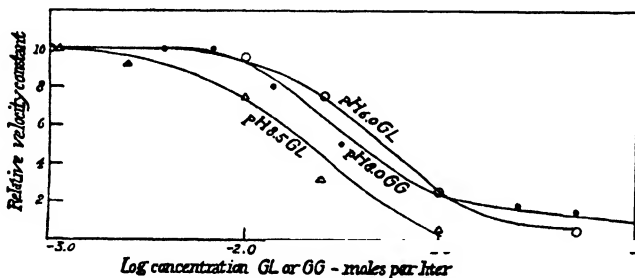


FIG. 1. Effect of concentration of glycyl leucine and glycyl glycine on velocity constant at various pH values.

out the experiments at as low a concentration as possible. The experimental limit for the methods used is about 0.01 M and this concentration was used throughout. In the case of glycyl glycine the effect of concentration was less marked. The results of the experiments on this point are shown in Fig. 1.

*Enzyme Concentration.*—von Euler and Josephson have noted that the inhibitory effect of glycine on trypsin is marked at pH 8.5 and negligible at pH 6.0 (7). Evidently then, if the enzyme preparation used contains such inhibitory substances, the more concentrated the enzyme solution the more the velocity of hydrolysis in the alkaline range will be slowed down compared to that at pH 6.0. This effect was in fact found to be marked with the glycerin extract if used direct or after partial dialysis but after the double dialysis described was too small to be detected. The presence of such effects can be tested for by diluting the enzyme with an inactivated portion of the same solution. The result of an experiment

with partially purified enzyme solution is shown in Fig. 2. The inactive enzyme markedly inhibits the reaction at pH 7 to 8 and has no effect at pH 6.

*Extent of Hydrolysis.*—It was found by Levene and Simms (8) that the hydrolysis of sarcosyl glycine, glycyl sarcosine, sarcosyl sarcosine, alanyl glycine, alanyl alanine, etc. did not go to completion but that there was an equilibrium between hydrolysis and anhydride formation. It is therefore necessary to consider the possibility that the position of the end-point depends on the pH. Experiments with excess enzyme, however, show that this is not the case but that the same final amount of hydrolysis takes place at all pH values. Complete hydrolysis occurs with all the peptides studied in this paper except glycyl d-l-leucine in which case the hydrolysis stops at 50 per cent, due either to anhydride formation or to the hydrolysis of only one isomer.

It was also found that fairly good monomolecular constants were obtained, except in the case of glycyl alanine which gave decreasing constants as the reaction

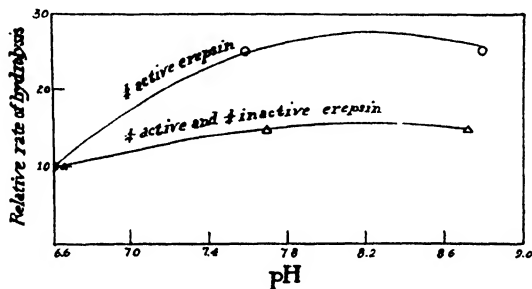


FIG. 2. Effect of concentration of erepsin on pH-activity curve.  $M/50$  biuret base.

progressed. This is probably due to the fact noted by von Euler (7) that alanine has a more marked inhibitory effect than the other amino acids. The fact that the constants did not decrease with time show that under the conditions used, inactivation of the enzyme is negligible.

As a result of these experiments it may be said that the monomolecular constants for the hydrolysis, when run with purified enzyme solution and with dilute substrate, are a measure of the effect of the pH on the reaction and are independent of the substrate concentration below the value used and of the enzyme concentration provided this is the same in the experiments compared.

In running the experiments a series of peptide solutions were made up and titrated with NaOH so as to differ by about 0.5 pH from each other over the range from pH 4.5 to 10.0. They were then allowed to come to the desired temperature in the water bath and the enzyme solution added. Samples were then taken at 0.5, 1, 2, 4 hours etc. until the reaction was about 25 per cent complete. The

velocity constant was then calculated from the increase in formol titration by means of the formula

$$C = \frac{1}{t} \log \frac{A}{A - X} \quad (1)$$

where  $A$  is the maximum increase noted with excess enzyme. The largest velocity constant so obtained was taken as 100 and the other constants expressed in terms of this. Six to twelve experiments were run with each peptide and the final figures obtained by averaging the separate values found at the same pH in different experiments.

The results of the experiments are shown graphically in Fig. 3. The experimental points are represented by circles the size of which shows approximately the magnitude of the probable error of the mean. The solid dots are from von Euler and Josephson (7) and agree well with the present figures. The curves were calculated by means of the assumption described below.

It is evident from the shape and position of the curves that the known equilibria in the substrate solutions are insufficient to account for the results. On the other hand, the fact that the optimum varies with the different peptides shows that the reaction depends to some extent on the condition of the substrate. If, however, the enzyme is assumed to be a weak acid or base with dissociation index ( $pK'$ ) of about 7.6 the curves may be predicted.

There are two possibilities either or both of which may lead to the pH optima found with the various substrates. Referring to the ionic species of the enzyme predominating above or below its dissociation index ( $pK' = 7.6$ ); or the ionic species of the substrates predominating above or below the indices given in Table II, it may be said that (I) the form of substrate predominating at low pH is acted on by the form of enzyme predominating at high pH, or that (II) the form of substrate predominating at high pH is acted on by the form of enzyme predominating at low pH, or that (III) both these reactions take place.

Other reactions must take place to a much smaller extent to agree with the observations.

The physical significance is clearer if the ionic species involved are considered, as summarized in Table I. In Table II it will be seen

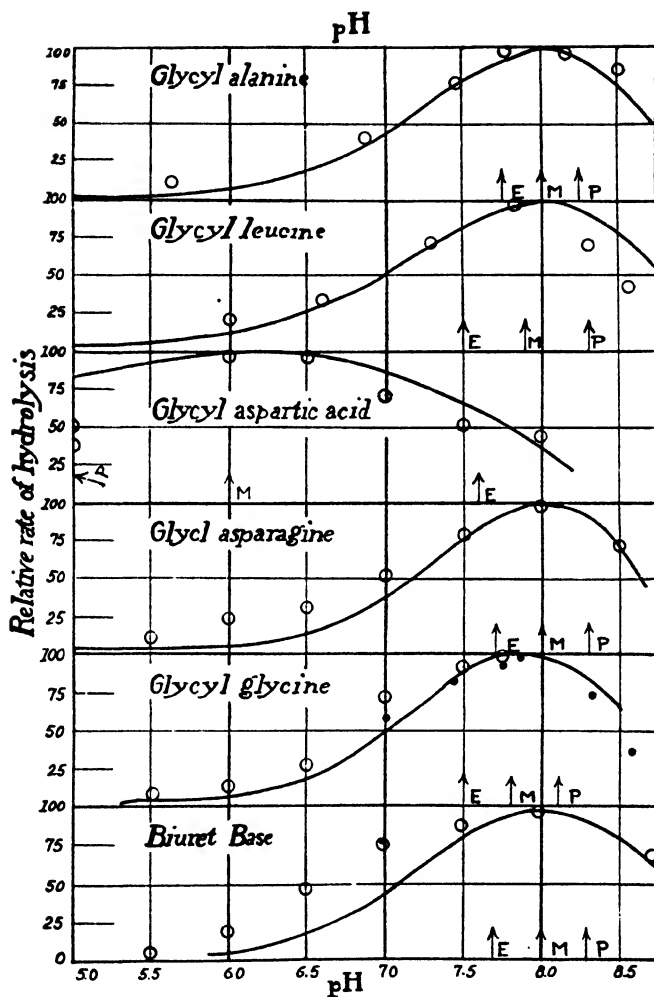


FIG. 3. Effect of pH on the rate of hydrolysis of various peptides.

○ observed.

— calculated.

The arrows indicate: pK, of substrate, *P*; the observed maximum, *M*; and the corresponding value of pK, (equation 7). Average pK, = 7.6.

that the four simple ampholytes change from "zwitterion" at lower pH to anion at higher pH (than the  $pK_s$ , involved in the optimum).

Glycyl aspartic acid, on the other hand, changes from zwitterion to zwitteranion at pH 4.45 which is the  $pK_s$ , involved in the optimum. However its change from zwitteranion to dianion at pH 8.60 is not accompanied by a noticeable change in reactivity

Biuret base, on the other hand, changes from cation to non-ionized molecule.

There is no evidence which permits a decision between assumption I and assumption II. According to I the reactive substrate forms have in common one ionized amino group—but so has the zwitteranion of glycyl aspartic acid which, according to this assumption, is not acted on (perhaps due to its negative charge if the enzyme is an acid).

TABLE II.

*Calculation of  $pK_s$  and Predominating Ionic Species at Low and High pH*

	Form pre dominating below $pK_s$	Form pre dominating above $pK_s$	$pK_s$	$H_{max}$	$pK_s$ calculated
GA	±	—	8.25	8.0	7.7
GL	±	—	8.29	7.9	7.5
GAp	±	±—	4.45	(6.0)	(7.6)
GApNH <sub>2</sub>	±	—	8.3	8.0	7.7
GG	±	—	8.07	7.8	7.5
Biuret base	+	0	8.3	8.0	7.7
Mean					7.6

According to assumption II the anions of the substrate would be acted on. But biuret base is an exception, it being non-ionized at high pH. If then a non-ionized amino group is necessary for reactivity glycyl aspartic acid is an exception.

It is equally uncertain whether the enzyme is a weak acid or a weak base. Neutral salts have an exponential negative effect on the reactions which according to Bronsted indicates reaction between two oppositely charged ions. However, the activity of weak bases and ampholytes has been shown to be anomalous (14) and the significance of the effect of neutral salt is doubtful.



The formulation of the reaction is the same on the basis of any of the above assumptions, the only difference being the significance attached to the various constants. It will be assumed for purposes of calculation that the enzyme is a weak base and that the reaction is between the negative monovalent ion of the peptides or the non-ionized form of the biuret base and the non-ionized form of the enzyme. Let

$$(HA) = \text{undissociated substrate}$$

$$(HE) = \quad \quad \quad \text{enzyme}$$

$$B = \text{total substrate} = A^- + (HA)$$

$$D = \quad \quad \quad \text{enzyme} = E^- + (HE)$$

Then

$$\begin{aligned} (HA) &\rightleftharpoons H^+ + A^- & (HE) &\rightleftharpoons H^+ + E^- \\ \frac{H^+ \times A^-}{(HA)} &= K_s & \frac{H^+ \times E^-}{(HE)} &= K_s \end{aligned} \quad (2)$$

and if the rate of reaction is assumed proportional to the product of the negative peptide ion and the non-ionized enzyme, then

$$-\frac{dB}{dt} = CA^- (HE) = \frac{C \cdot D \cdot K_s H^+ B}{(K_s + H^+) (K_s + H^+)} \quad (3)$$

and at constant substrate concentration and pH

$$\frac{C \cdot D \cdot K_s H^+}{(K_s + H^+) (K_s + H^+)} = \frac{1}{t} \log \frac{B_0}{B} = \text{velocity constant} \quad (4)$$

The observed velocity constant will therefore be expressed by the equation

$$C' = \frac{CK_s H^+}{(K_s + H^+) (K_s + H^+)} \quad (5)$$

and the position of the maximum velocity can be obtained by differentiating this expression with respect to  $C'$  and  $H$ . Setting  $dC/dH$  equal to 0 and solving for  $H_{max}$ , the following expression is obtained

$$H_{max} = \sqrt{K_s K_s} \quad (6)$$

or

$$pH_{max} = \frac{pK_s + pK_s}{2} \quad (7)$$

Since the values of the dissociation indices  $pK_s$  of the substrates are known it is now possible to solve for the value of the dissociation index of the enzyme  $pK_s$ . The results of this calculation are shown in Table II. The calculation gives a fairly constant value for  $pK_s$  of about 7.6 (*i.e.*,  $K_s = 10^{-7.6}$ ). In order to obtain the entire curve this value of  $K_s$  may now be substituted in (5) and the equation solved for the value of the velocity constant  $C'$ . In order to compare these figures with those found experimentally it is necessary to express them as per cent of the maximum value. The maximum value found for  $C'$  is therefore taken as 100 and the other values calculated in proportion. These figures at the different pH were then used to plot the solid line shown in Fig. 3.

The calculation made above shows that the assumptions used are sufficient to account for the general shapes and position of the curve but involve several constants and are not very convincing in the absence of independent evidence. If the enzyme really has a dissociation index of about 7.6 it should be possible to show that some other property changes at that pH. It was found by Goulding, Borsook and Wasteneys (9) that the effect of the pH on the inactivation of pepsin agreed with the assumption that the enzyme was a weak electrolyte with a dissociation constant near pH 6. It seemed possible that a similar effect could be noted in the case of erepsin. The rate of inactivation of erepsin was therefore determined at various pH. The experiment was carried out in two ways, by determining the amount of enzyme in the solution after varying lengths of time by means of the rate of hydrolysis of glycyl glycine and also by following the decrease in the rate of hydrolysis of glycyl glycine with time at 35°C.

*First Method.*—A series of tubes containing the purified erepsin solution and 0.01 M glycine were adjusted to pH values about 0.5 pH units apart over the range of from pH 5 to 10 and put at 25°C. 1 cc. samples were removed at daily intervals and added to a stock solution of 0.1 M glycyl glycine pH 7.8. The rate of hydrolysis of the glycyl glycine and the velocity constant were then determined as usual and the concentration of enzyme assumed to be proportional to the velocity constant. The values obtained in this way for the concentration of enzyme were then plotted against time and the rate of inactivation of the enzyme calculated. The process was found to be approximately monomolecular. The velocity constants of these curves were then assumed proportional to the rate of inactiva-

tion of the enzyme. Inactivation proceeded slowly up to pH 6.5. It then became more rapid becoming constant above pH 9. The sample at pH 9 was half inactivated in about 3 days. This value was then taken as 100 and the other values expressed in proportion.

*Second Method.*—The hydrolysis of glycyl glycine under conditions such that the enzyme concentration remains unchanged is known to be monomolecular and the velocity constants are proportional to the enzyme concentration. If the experiment is arranged so that the enzyme is being inactivated the velocity constants when calculated for succeeding time intervals will drop and the values for each time interval may be assumed proportional to the average concentration of enzyme over that interval. This procedure furnishes a method therefore for determining the destruction of the enzyme in the presence of the substrate. In order to obtain significant results, however, it is necessary to arrange the experi-

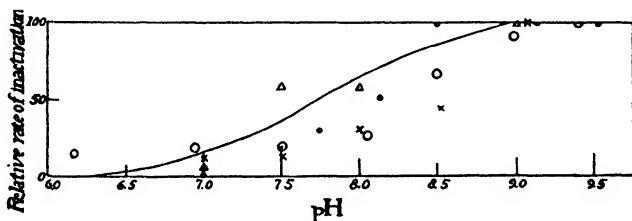


FIG. 4. Inactivation of erepsin at various pH.

— curve calculated from  $pK_a = 7.7$ .

○ inactivation at  $35^\circ + M/100$  glycine.

△ " "  $25^\circ + M/50$  glycyl glycine experiment (1).

● " " " " " " (2).

× " " " " " " (3).

ment in such a way that the enzyme is inactivated before the substrate is hydrolyzed to a great extent (10). These conditions are fulfilled by carrying on the reaction with dilute enzyme and concentrated substrate and at a rather high temperature. A series of solutions  $0.1 M$  glycyl glycine were therefore adjusted about  $0.5$  pH apart over the range of from 5 to 10 and placed at  $35^\circ C$ . The erepsin was added and the hydrolysis followed. A smooth curve was drawn through the figures obtained in this way and the monomolecular constants calculated for short time intervals. These were assumed to be proportional to the concentration of enzyme present at the corresponding time interval. The rate of inactivation of the enzyme could therefore be determined from them as described above.

The effect of the pH on the inactivation of erepsin as determined by these methods is shown graphically in Fig. 4. The solid line rep-

resents the calculated results assuming that the form of the enzyme present in acid solution is very stable compared to the form that is present in alkaline solution and that the value of the dissociation constant is  $10^{-7.6}$ .

As before it does not matter whether the enzyme is considered as a weak base or acid. The experimental points are irregular but lie in the region of the curve and a little to the alkaline side. The agreement is as good as can be expected for this type of experiment and furnishes independent evidence for the assumption made before that the enzyme had a dissociation curve near pH 7.6.

*Calculation of the Rate of Inactivation.*

Assume that the enzyme is a weak acid and that the negative ion is very unstable compared to the non-ionized form then

$$\begin{aligned} H^+ + E^- &\rightleftharpoons (HE) & D &= \text{total enzyme} \\ H^+ \times E^- &= K_s(HE) & D &= E^- + (HE) \\ E^- &= \frac{K_s(HE)}{(H^+)} & HE &= (D - E^-) \end{aligned} \quad (8)$$

and

$$E^- = \frac{K_s D}{K_s + H^+} \quad (9)$$

If the rate of inactivation is proportional to the amount of the ionized form then at any constant pH

$$\frac{-dD}{dt} = C' E^- = \frac{C' K_s D}{K_s + H^+} \quad (10)$$

or

$$\frac{C' K_s}{(K_s + H^+)} = \frac{1}{t} \log \frac{D_0}{D} = \text{velocity constant}$$

The reaction will therefore be monomolecular at any pH and the observed velocity constant will be given by the formula

$$C'' = \frac{C' K_s}{(K_s + H^+)} \quad (11)$$

Substituting the value  $10^{-7.6}$  for  $K_a$  and solving for the value of  $C''$  at different values of  $H$ , a series of values are obtained which should be proportional to the observed inactivation constants. In order to compare them the maximum constant value found as the value of  $H$  becomes less, is taken as 100 and the values at the other pH expressed in proportion. The solid line in Fig. 4 has been plotted from these figures.

### *Effect of Neutral Salts.*

It was stated above that the results agreed equally well with several assumptions in regard to the ionic species taking part in the reaction. According to Brönsted's (11) theory of chemical reaction velocities the effect of neutral salts on the rate of a reaction differs depending on whether the reaction is between ions of different or like sign or between an ion and a molecule.

The effect of neutral salts on the reaction is complicated by the fact that the presence of salts causes precipitation unless special precautions are taken. This probably accounts for the contradictory literature on the subject (12). The dialyzed glycerin extract becomes cloudy below pH 5 and this precipitation is prevented by the addition of neutral salts. Under these conditions neutral salts slightly increase the rate of hydrolysis.  $\text{CaCl}_2$  on the other hand, causes a slight precipitate on the alkaline side of pH 7 and in this range retards the hydrolysis. These effects are presumably secondary and appear to be connected with the formation of the precipitate. It was found by Mr. Johnston that if the erepsin solution was dialyzed in the presence of 0.10 M  $\text{CaCl}_2$  and then filtered a solution was obtained which remained clear over the range covered by the experiments. It was less active than the usual preparation. The effect of 0.10 M  $\text{CaCl}_2$  on the hydrolysis of 0.01 M GG at various pH using this erepsin solution dialyzed in the presence of  $\text{CaCl}_2$  is shown in Fig. 5. The salt inhibits at all reactions but the effect is much more marked on the alkaline side of pH 7 so that the presence of the salt causes a marked shift in the optimum. This result had been obtained by Abderhalden and Fodor (13).

The effect of increasing concentrations of NaCl and  $\text{CaCl}_2$  on the rate of hydrolysis of glycyl glycine at pH 7.0 was then determined

with the erepsin prepared as described above. The result is given in Fig. 6 in which the relative rate of hydrolysis has been plotted against the log of the salt concentration. There is evidently a negative exponential salt effect which is Brönsted's prediction for a reaction between two ions of opposite charge. The only assumption in regard to the mechanism that predicts this is that the enzyme is a weak base and that the reaction is between the negative dipeptide ion and the positive enzyme ion. There is probably some doubt, however, as to the application of Brönsted's theory to catalytic reactions and the experiment can hardly be considered conclusive, especially since amines and ampholytes show anomalous changes in activity in the presence of neutral salts (14), particularly those of the type of  $\text{CaCl}_2$ .

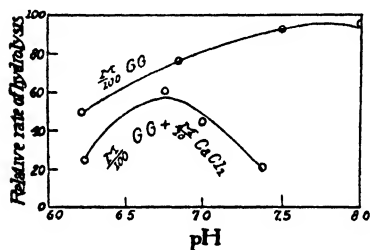


FIG. 5.

FIG. 5. Effect of  $m/10 \text{ CaCl}_2$  on rate of hydrolysis of  $m/100$  glycyl glycine at various pH. Erepsin dialyzed in  $m/10 \text{ CaCl}_2$ .

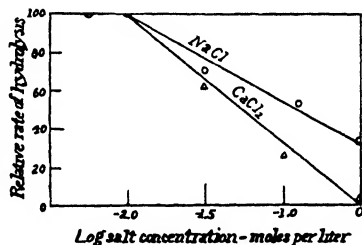


FIG. 6.

FIG. 6. Effect of  $\text{CaCl}_2$  and  $\text{NaCl}$  on hydrolysis  $m/100$  glycyl glycine pH 7.0.

#### *Calculation of the Relative Rate of Hydrolysis of Different Substrates.*

A number of workers have made determinations of the relative rate of hydrolysis of various peptides by erepsin, *i.e.*, the specificity of the enzyme. These determinations have, as a rule, been made at the same pH. It is evident, however, that if the mechanism assumed in this paper is correct, the determination of the relative rate of hydrolysis of different peptides is a difficult matter, except in the case where the pH-activity curves are the same for the different substrates. In general, however, it would be necessary to correct the observed rate for the fraction of the total enzyme and of the total substrate active at the pH used. The extent of this correction would vary

enormously depending on which of the various possible assumptions are used. In addition it must be shown that the relative rate of hydrolysis of the substrates compared is independent of the concentration. According to the experiments reported in the first part of the paper this is only true in very low concentrations. In view of these considerations it appears impossible, without further information, to make a significant comparison of the rates of hydrolysis of various peptides.

#### SUMMARY.

1. The rate of hydrolysis at different pH values of glycyl glycine, glycyl leucine, glycyl alanine, glycyl asparagine, glycyl aspartic acid and biuret base has been determined.

2. The pH-activity curves obtained in this way differ for the different substrates.

3. The curves can be satisfactorily predicted by the assumption that erepsin is a weak acid or base with a dissociation constant of  $10^{-7.6}$  and that the reaction takes place between a particular ionic species of the enzyme and of the substrate. There are several possible arrangements which will predict the experimental results.

4. The rate of inactivation of erepsin at various pH values has been determined and found to agree with the assumption used above, that the enzyme is a weak acid or base with a dissociation constant of about  $10^{-7.6}$ .

5. It is pointed out that if the mechanism assumed is correct, the determination of a significant value for the relative rate of hydrolysis of various peptides is a very uncertain procedure.

Most of the experimental work reported in this paper was done by Mr. Frank F. Johnston.

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## THE PERMEABILITY OF DRY COLLODION MEMBRANES. II.

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When freshly made collodion membranes are immersed in water before the complete removal of the organic solvents, the membranes are permeable for all substances of small molecular weight. If the membrane is allowed to dry, however, either before or after placing in water, the permeability to electrolytes is lost and the membranes exhibit in general the same type of permeability as living cells (1-3). They furnish therefore an interesting model of the cell in that the permeability of the "wet" membranes is similar to the dead cell while that of the "dry" membranes is similar to the living cell. The mechanism of the permeability of the "wet" membranes was firmly established by the work of Duclaux and Errera (4) who found that the relative rate of flow of various liquids through these membranes was in proportion to the viscosity of the liquids. There seems no doubt therefore that these membranes consist of a network of capillaries through which the solutions pass. Hitchcock (5) and Bjerrum and Manegold (6) have been able to calculate the size of these pores as of the order of magnitude of  $10^{-6}$  cm. As the percentage of water held in the membrane decreases the size of the pores decreases until with membranes containing 5 per cent or less water the rate of flow of water is too small to be measured and the pore size cannot be determined. Collander has found that semipermeability becomes more marked as the percentage of water decreases and assumes as does Michaelis (7) that this selective action of the dry membranes is due to the fact that the pores become too small to allow the passage of large molecules. The impermeability to electrolytes is accounted for by Michaelis (8) as due to the negative charge on the membrane which prevents the passage of negative ions while secondary differences in the rate of passage of the ions are ascribed to

differences in the degree of hydration of the ions. The potential differences observed by Michaelis are accounted for as diffusion potentials caused by the great differences between the mobilities of the anion and cation in the pores of the membrane. An alternative hypothesis is that the pores disappear in the dry membrane and that substances pass by dissolving in and diffusing through the collodion. The essential difference between the two points of view consists in that from the point of view of pores, molecules in solution are surrounded by an atmosphere of the solvent molecules and really move relative to them, the membrane simply serving to alter the area and length of the column of solvent through which the solute can diffuse. The permeability is therefore determined by the diffusion coefficient in the solvent and by the effective diffusion area of the membrane.

From the point of view of solution the molecules are considered to diffuse through the material of the membrane and the solvent in contact with the membrane affects the permeability only indirectly, in that the concentration of the solute in the membrane depends upon the partition coefficient of the substance between the membrane and the solvent. In the case of diffusion of substances in solution the results can be predicted at least qualitatively by either point of view, although there is evidence in favor of the solution idea. The simplest case appears to be that of the passage of gases; and in this case, as was pointed out in a preliminary paper (3), the results are more in accord with the idea of solution. It may be mentioned that there are undoubtedly cases where passage takes place through pores, as in the wet collodion membrane or in unglazed porcelain, while there are also cases such as the passage of solutes through a layer of ether or of gases through rubber (9) or metals where the process is one of solution.

### *Experimental Procedure.*

*Preparation of Membranes.*—5 cc. of Merck's U. S. P. collodion was placed in a  $1.5 \times 15$  cm. test-tube and rotated mechanically in a horizontal position for 20 minutes. The tubes were then allowed to dry for 3 days. They were then filled with water and the membranes removed, drained and dried for 3 days in a desiccator over  $H_2SO_4$ . The membranes could be made thicker or thinner by using more or less collodion. Thinner membranes frequently have imperfections and the thicker ones render the experiments slow.

*Attachment of the Membrane.*—The glass tube to which the membrane was attached was just large enough so that the membrane could be drawn over it smoothly without wrinkling. The tube was first covered with a thin coat of vacuum stop-cock grease and the membrane slipped on. It was then bound firmly in place with rubber bands.

*Determination of the Rate of Passage of Gases.*—The determination of the permeability for gases was made in the apparatus shown in Fig. 1. The membrane and glass tube were completely filled with gas by running the gas first into the membrane and then into the outside tube, by appropriate manipulation of the stop-cocks. The volume of gas passing through was read from the movement of

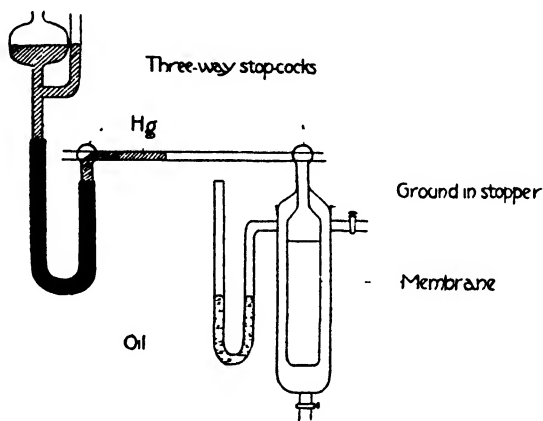


FIG. 1. Apparatus for measuring rate of passage of gases through collodion membranes.

the mercury meniscus and checked by the change in the oil manometer. Since the total volume of the apparatus is large compared to the volume of gas passing through, the oil manometer is very sensitive to changes in barometric pressure and temperature. The entire apparatus was therefore immersed in a large constant temperature water bath. Even under these conditions, unless great precautions are taken against leaks, etc., the change in the outside manometer did not agree quantitatively with that of the inside one.

*Determination of the Solubility of Gases.*—The volume of gas taken up by collodion was determined in an apparatus essentially the same as that described by Lefebure (10). The apparatus containing the collodion was evacuated to less than a millimeter of pressure for several hours before use.

*Determination of the Permeability to Solutes.*—A glass tube held in a cork was tied in the end of the membrane and the membrane containing the solution sus-

pended in a test-tube of water. The quantity of solute outside was determined by titration with alkali in the case of acids or by  $\text{AgNO}_3$  in the case of chlorides. Phenol was determined by titration with iodine.

*Determination of the Solubility of Acetic Acid.*—Several grams of collodion membrane were placed in a measured volume of the solution and allowed to remain until no further change in concentration of the solution was noted. This required from a day to several weeks depending on the concentration of the solution and the thickness of the membrane. The collodion was then removed from the solution, rinsed with water and placed in a small volume of water. It was placed in fresh water every day until no further solute was found in the water. The wash waters were then combined and the total amount of solute given off from the collodion determined. This total amount taken up by the collodion was also determined by difference, from the analysis of the original solution before and after the collodion had been placed in it. The figures obtained agreed with each other, showing that the process is reversible. This is not true of  $\text{NH}_3$  which forms an irreversible compound after long standing.

*Effect of the Size and Thickness of the Membrane and the Time of the Experiment.*

From either the point of view of diffusion through pores or of solution and diffusion through the membrane, it would be expected that the general form of the equation governing the process would be that of Fick's diffusion law, which states that the quantity of material passing in unit time is proportional to the area and to the concentration difference across the membrane. The significance of the terms of the equation is different, however, depending on which assumption is used.

*Assumption I. Diffusion Takes Place through Pores.*

If the experiment is arranged so that there is a "steady state," that is, so that the concentration difference across the membrane is constant, the concentration gradient across the (homogeneous) membrane is linear and the equation may be written

$$Q = \frac{P t A (C_1 - C_2)}{h} \quad (1)$$

or

$$P = \frac{Q h}{t A (C_1 - C_2)}$$

in which  $Q$  is the quantity of substance that passes through area  $A$ , of thickness  $h$ , in time  $t$  when the concentration difference in the solution

or gas on the opposite sides of the membrane is  $(C_1 - C_2)$ .  $P$  may be called the permeability constant, since it is the amount of material that will pass through unit area and thickness of membrane in unit time under unit difference of concentration (or pressure). This relation was found to hold both with gases and with substances in solution. The partial pressure of the gas was used. The amount of substance passing at the beginning of the experiment will evidently be smaller than that at the steady state since some of the substance remains in the membrane, so the rate increases slowly at first and then remains constant. The experiments were continued until this constant rate was reached. This required an hour or so for the gases and several days for some of the substances in solution.

*Dimensions of  $P$ .*—It will be noted that if the area and thickness are expressed in the same units and the pressure or concentration in terms of quantity per unit volume,  $P$  then has the dimensions: *area over time*. Since the mole fraction, which is the significant figure, of the molecular species present in small amount (or the pressure of a gas), is nearly proportional to the mass per unit volume, the concentrations of gases and solutes in low concentration may be expressed as quantity per unit of volume. In these experiments the results are expressed as  $\text{cm.}^2$  per day. The following is an example of the calculation.

$$A = \text{area membrane} = 72 \text{ cm.}^2$$

$$h = \text{thickness membrane} = 26 \times 10^{-4} \text{ cm. (calculated from area and weight)}$$

$$t = 2 \text{ days}$$

$$Q = 2.2 \times 10^{-3} \text{ mole}$$

$$C_1 = \frac{10 \times 10^{-6} \text{ mole}}{\text{cm.}^3}$$

$$C_2 = 0$$

$$P = \frac{2.2 \times 10^{-3} \text{ mole} \times 26 \times 10^{-4} \text{ cm.}}{72 \text{ cm.}^2 \times \frac{10 \times 10^{-6} \text{ mole}}{\text{cm.}^3} \times 2 \text{ days}} \quad (2)$$

$$= 0.040 \text{ cm.}^3 \text{ per day.}$$

Evidently it makes no difference what units are used to express the quantity and concentration as long as the units are the same in both cases. In the case of a gas, grams or cc. per  $\text{cm.}^3$  at standard pressure and temperature, can be used. In a solution, however, the mole

fraction of the *solvent*, *i. e.* the molecular species present in excess, is not proportional to its volume concentration, and in this case the dimensions of  $P$  cannot be reduced to area over time but must be expressed as quantity per unit membrane size per unit of time and unit of osmotic or vapor pressure, or whatever property of the solvent is assumed to be the determining factor.

From the point of view of pores the value of  $P$  is the diffusion coefficient of the substance in water or in whatever substance is supposed to fill the pores of the membrane. It differs from that determined in water without a membrane because it has been expressed per unit area of membrane whereas the area and length of the pores only should have been used. It follows from this point of view that the permeability is a function of the diffusion coefficient of the substance in the solvent and of the effective pore area and length, so that the membrane affects the value only by changing the effective diffusion area. Evidently then the rate of diffusion of the same substance should be different depending on the solvent. A gas for instance should diffuse much more rapidly through a membrane in the gas, in which case the pores are filled with the gas, than through the same membrane immersed in water since the pores would now be filled with water and the rate of diffusion of gases in gases is some 10,000 times as great as the rate of diffusion of gases in water. As will be seen, this is not the case. It would also be predicted that the relative rates of penetration should be in the same order as the diffusion coefficients in water, but the differences should be magnified since the percentage of the total number of pores through which small molecules can pass is greater than that for large molecules. In general this is true, but there are marked exceptions since electrolytes do not pass with appreciable velocity whereas some large molecules, as phenol, pass rapidly. In the case of large molecules having a higher rate of penetration than small ones it is necessary to assume that the diffusion law does not hold in small capillaries or that the substance becomes concentrated in the capillaries.

*Assumption II. The Substances Dissolve in and Diffuse through the Material of the Membrane.*

The equation for the rate of penetration is the same as before but from this point of view the terms referring to the dimensions of the

membrane are correct but the concentration terms must now refer to the concentration in the two sides of the membrane instead of the concentration in the solution.\* If  $S$  is the partition coefficient of the substance between the solvent and collodion, *i. e.*

$$S = \frac{\text{Concentration in collodion}}{\text{Concentration in solvent}} \quad (3)$$

then the concentration in the collodion is  $SC$  and equation (1) may be written

$$Q = \frac{t D A S (C_1 - C_2)t}{h} \quad (4)$$

or

$$D = \frac{Q h}{t A S (C_1 - C_2)}$$

$$= \frac{P}{S}$$

$D$  has now the same dimensions as  $P$  in the previous case but is of different significance, since it is the diffusion coefficient of the substance in the collodion (more strictly, in a saturated solution of whatever substances are present in the collodion). From this point of view differences in  $Q$ , the quantity passing through, are due to differences in the partition coefficient of the substance between the solvent and collo-

\* It is assumed, in accord with the work of Noyes and Whitney and others on solution of solids, that equilibrium exists at the interface and that the time element consists in the diffusion. This is a reasonable assumption since the actual interface is of molecular dimensions and the activity of a substance at any point in a solution would differ very slightly from that at another point distant by a few molecular diameters. Unless there were some special block at the interface the activity on the two sides would therefore be expected to be practically identical, *i. e.*, there would always be equilibrium at the interface.

† If the solutions on the two sides are different the partition coefficients will be different and the general equation is

$$Q = \frac{t D A (S_1 C_1 - S_2 C_2)}{h}$$

This is the case in the experiments reported by Irwin (17).

dion and to the diffusion coefficient of the substance in collodion. This value,  $D$ , should therefore depend only on the substance and should be independent of the material surrounding the membrane. Gases therefore should give the same value for  $D$  when the membrane is surrounded by gas as when it is in water, and this is the case. The

TAB  
*Permeability of Col*

Substance	H <sub>2</sub>	H <sub>2</sub>	NH <sub>3</sub>	H <sub>2</sub> O	N <sub>2</sub>	O <sub>2</sub>
System measured	$\frac{H_2}{H_2}$	$\frac{H_2}{H_2O}$	$\frac{NH_4OH}{H_2O}$	$\frac{H_2O}{\text{Sugar}}$	$\frac{N_2}{N_2}$	$\frac{O_2}{O_2}$
Moles per liter or pressure at	0.05-1.0	1.0	0.10		0.05-20.0	0.05-2
P = permeability = $\frac{\text{cm}^3}{\text{day}} \times 10^4$	7.5	7.3	1.2		45	1.1
$S = \frac{\text{gm per cc collodion}}{\text{gm per cc water or gas}}$	10	10	2.0	0.7	15	23
D = Diffusion coefficient in collodion = $\frac{P}{S} = \frac{\text{cm}^2}{\text{day}} \times 10^4$	75	73	0.6	30-100	3.0	4.8
Molecular weight	2	2	17	18	28	32
Molecular radius $\times 10^8$ cm.	1.4	1.4		1.44	1.57	1.45

values for the permeability should have no general relation to the molecular weight or size except in so far as the partition coefficient between water and collodion varies with these quantities. The values of  $D$  should decrease as the size of the molecule increases, although there is no known relation between the diffusion coefficient and the molecular diameter or weight except when the molecule of solute is large compared to that of the solvent. In that case the diffusion coefficient is



inversely proportional to the diameter of the molecule or to the cube root of the molecular weight (11). Empirically it is known that in water the diffusion coefficient of molecules of the same order of size as the water molecule is inversely proportional to the square root of the molecular weight. In the present case the molecules of the solvent,

## E 1.

*dion Membranes.*

HCl gas	HCl	CO <sub>2</sub>	CO <sub>2</sub>	CO <sub>2</sub>	HCOOH	CH <sub>3</sub> COOH	CH <sub>3</sub> NH <sub>2</sub> COOH	C <sub>2</sub> H <sub>5</sub> OH	CH <sub>3</sub> ClCOOH	CHCl <sub>2</sub> COOH	HgCl <sub>2</sub>
$\frac{\text{HCl}}{\text{HCl}}$	$\frac{\text{HCl} - \text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{CO}_2}{\text{CO}_2}$	$\frac{\text{CO}_2 - \text{H}_2\text{O}}{\text{CO}_2 \text{ H}_2\text{O}}$	$\frac{\text{CO}_2}{\text{O}_2}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$
1.05-.10	0 1	0 05-1	1	1	0 1	0 01-1 0	0 10	0 2	0 1	0 1	0 1
240	0	6 7	8 0	7 0	.16	.038	0	.15	.025	.0033	.003
>100	0	4 6	4 6	4 6	.75	1 0	0	2 8	36	.11	16
2.4?	?	1 4	1 7	1 5	.21	.038	?	.054	.07	.03	.018
36		44			46	60	75	90	94	128	270
1.59		1 61									

collodion, are much larger than those of the solute, and it might be expected that the rate of diffusion would vary as some higher power of the molecular weight.

*Experimental Results.*

A summary of the results is given in Table I, in which the substances have been arranged in order of their molecular weight. The permea-

bility decreases in an irregular way as the molecular weight increases but there are marked exceptions. HCl gas for instance passes many times more rapidly than hydrogen; and phenol, although of large molecular weight, passes many times more rapidly than acetic acid; while amino acetic does not pass with measurable velocity. The clearest results are those for the gases, since the theory of the passage of gases through fine openings is well worked out and there is less chance for secondary complications. It is known that under all conditions the relative volume of gas passing through a small opening is nearly inversely proportional to the square root of the density of the gas, and that this relation holds even when the capillary is of the order of magnitude of the mean free path of the gas molecule (12). Hydrogen should therefore pass the most rapidly and CO<sub>2</sub> the most slowly. CO<sub>2</sub> and H<sub>2</sub>, however, pass at the same rate while O and N pass much more slowly and HCl goes very much more rapidly. On the other hand, it can hardly be assumed that CO<sub>2</sub> and HCl can pass through pores that will not admit H<sub>2</sub> and the other gases, since the relative diameters of the gas molecules are known and do not differ much, CO<sub>2</sub> being the largest. (There are several independent methods of determining the molecular radius, which lead to different results. The relative order of size, however, is the same for all methods; cf. Loeb (12).) On the other hand, when the results are expressed as coefficients of diffusion in collodion, the results are correctly predicted qualitatively for all the substances measured, without any supplementary hypotheses as to electrical effects, etc. Those substances that do not pass the membrane do not dissolve in it, and those like phenol, which are exceptions from the point of view of pores, are found to be soluble. The most striking examples of the effect of solubility, such as NH<sub>3</sub> gas, H<sub>2</sub>S gas, and to a certain extent HCl and the collodion solvents of higher molecular weight, cannot be tested from the point of view of solubility since they either destroy the membrane or combine with it irreversibly so that the solubility coefficient cannot be determined. Collodion was found to take up large quantities of NH<sub>3</sub> and H<sub>2</sub>S, but the membrane changes color and becomes brittle while only a small part of the gas can be removed. Evidently a chemical reaction takes place subsequent to solution.

TABLE II  
*Effect of Water on Passage of H<sub>2</sub> through Collodion*

$$\text{Membrane factor} = \frac{h}{A} = 2 \times 10^{-5}$$

Membrane Outside	Dried over H <sub>2</sub> SO <sub>4</sub> Dry air 1 atmosphere	Saturated with H <sub>2</sub> O vapor Air saturated with H <sub>2</sub> O 1 1 atmosphere	Immersed in H <sub>2</sub> O H <sub>2</sub> O, air bubbled through
Inside	Dry H <sub>2</sub> , 1 1 atmosphere	H <sub>2</sub> saturated with H <sub>2</sub> O 1 1	H <sub>2</sub> , 1 1 atmosphere
Partial pressure H — atmosphere	1 1	1 1	1 1
Cc H <sub>2</sub> , per hr	2 2	2 3	2 05
$P = \frac{\text{cm}^3}{\text{day}} \times 10^4$	9 6	10 0	9 0
Order of running experiment	1	2	3

The diffusion coefficients of the substances in collodion increase as the molecular weight decreases, which is a reasonable result. The increase, however, is much more rapid than would be expected from the relative rates of diffusion in water, and although no theoretical relation is known between the diffusion coefficient and the molecular weight when the solute molecule is smaller than that of the solvent it appears hardly probable that the diffusion coefficient should increase as rapidly as the figures show. It will be noted, however, that those substances having a molecular weight of 60 and above give diffusion coefficients of about the relative order expected from the molecular weight, while those for O, CO<sub>2</sub> and N are also consistent with each other, as are the values for water and hydrogen. It might be supposed from these results that there are some very small pores and that while the large molecules pass by solution and diffusion the small molecules can also pass through pores. This assumption, however, appears to be ruled out by the fact that H and CO<sub>2</sub> pass at the same rate whether the membrane is immersed in water or in the dry gas.

The result of such an experiment with hydrogen and either a dry membrane or membrane immersed in water is shown in Table II. The rate of passage of the hydrogen is the same within the error of the readings. This is difficult to account for if it is assumed that the hydrogen passes through pores, since, when the membrane is surrounded by gas, the pores must be filled with gas molecules, while when the membrane is in water the gas must diffuse through water, and it is known that the rate of diffusion of gases in water is about 1/10,000 the rate of diffusion of gases in gases. It can hardly be supposed that the pores allow the hydrogen to enter but not the water since the hydrogen molecule is very slightly smaller than the water. Neither can it be assumed that the pressure forced the water out of the pores since the pressure necessary to force water out of such small pores would be many hundreds of atmospheres. From the point of view of solution and diffusion the result is exactly what is expected since the difference in partial pressure of the hydrogen on the two sides of the membrane is 1.1 atmospheres in both cases and the rate of diffusion through the collodion should therefore be the same.

Table III gives the result of a similar experiment with CO<sub>2</sub>. In this case the amount of gas passing the membrane was determined by

titration in the outside solution instead of by loss of volume on the inside, so that there is no doubt that the measurement represents the

TABLE III.  
*Diffusion CO<sub>2</sub>, Membrane in H<sub>2</sub>O.*

	Outside			H <sub>2</sub> O sat. + CO <sub>2</sub>		
	Inside	H <sub>2</sub> O + M/100 NaOH.		Time required to bring to pH 8.0 determined		
	Membrane No.	2		4		
F × 10 <sup>3</sup>		2	8	2.8		CO <sub>2</sub> /CO <sub>2</sub>
Cc. M/100 NaOH	1	1	1	1		
≈ cc. CO <sub>2</sub>		.224		.224	.23	
Δt, hrs. → pH 8.0		.17	.18	.26	.21	
Average		.17		.22		
Cc. CO <sub>2</sub> /day/at	31			24		
P = cm. <sup>2</sup> /day × 10 <sup>4</sup>	8 6			7.4		6.7

CO<sub>2</sub> diffusion coefficient in H<sub>2</sub>O = 1.2 cm.<sup>2</sup>/day

in O<sub>2</sub> = .18 cm.<sup>2</sup>/sec. = 1.6 × 10<sup>4</sup> cm.<sup>2</sup>/day

TABLE IV.

*Effect of Water on Permeability of Membrane with Small Holes.*

Membrane filled with air under 3 cm. Hg pressure.

8 punctures made in membrane with fine glass needle and rate measured in air and in water.

	Membrane in air	Membrane in H <sub>2</sub> O
Cc. per hr.....	4000	0.3

TABLE V.

*Effect of Area and Method of Preparing Membrane on Quantity of CO<sub>2</sub> Absorbed.*

Method of preparing membrane.....	Poured into water	Membrane made in test-tubes	Membrane made on mercury	
Area membrane per gm. collodion. cm <sup>2</sup> .....	?	1100	120	50
Cc. CO <sub>2</sub> per cc. collodion 760 mm. pressure ..	4.8	5 1	4.7	4.7
	4.7			
	4.3			

amount of gas passing through the membrane. Since the radius of the CO<sub>2</sub> molecule is known to be as large or larger than that of water it cannot be assumed that the water was unable to enter the pores.

The experiment was controlled by measuring the rate of diffusion of air through a membrane which had been punctured with a fine glass needle. The result of this experiment is shown in Table IV. The gas now passes more than 10,000 times as fast when the membrane is in air as when it is in water.

The foregoing experiments agree qualitatively at least with the assumption that the passage of substances is governed by solution rather than by diffusion through pores. It is possible, however, to obtain confirmatory evidence from several different experiments.

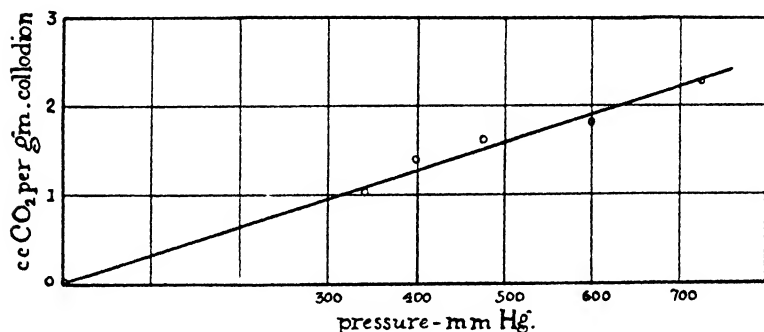


FIG. 2. Solubility of  $\text{CO}_2$  in collodion at different pressures.

*Effect of Pressure or Concentration and Form of Membrane on the Amount of Substance Dissolved in Collodion.*

If the substances form a solution in the collodion it would be expected that the quantity dissolved would be proportional to the concentration or pressure of the substance and independent of the surface of the collodion. Table V shows that the amount of  $\text{CO}_2$  absorbed by collodion is independent of the area of the collodion and is nearly the same whether the collodion is prepared by pouring into water or made in the form of a dry membrane. The solubility is a little less in the water sample and it was noted also that some samples of collodion showed a slightly different value for the solubility. Collodion is known to be a mixture and it is possible that the solubility in the various constituents is different, so that different samples would be

expected to vary slightly. If this were the case it might account for the rapid decrease of the diffusion coefficient with increasing molecular weight. But it must be assumed that the membrane is heterogeneous in the plane *perpendicular* to the direction of diffusion.

The amount of CO<sub>2</sub> dissolved by collodion at different pressures is shown in Fig. 2. The quantity is proportional to the pressure up to pressures of 760 mm. Hg.

TABLE VI.  
*Solubility of Acetic Acid and Phenol in Collodion.*

Concentration in H <sub>2</sub> O	Concentration in collodion		Partition coefficient = $\frac{\text{Concentration in collodion}}{\text{Concentration in H}_2\text{O}}$	
Acetic acid or phenol	Acetic acid	Phenol	Acetic acid	Phenol
mm per cc	mm per cc	mm per cc		
0.01	0.01		1.0	
0.25		0.071		2.8
0.5		(20)		(4.0)
10	0.99	28	1.0	2.8
20		57		2.8
1.0	87		87	
5.0	3.05		61	

TABLE VII.  
*Solubility of Acetic Acid in Collodion from Acetic Acid-Sodium Acetate Mixtures*

Cc N/10 HA	10	8	5	2	0
Cc N/10 NaA	0	2	5	8	10
Moles HA per cc collodion					
Moles HA per cc H <sub>2</sub> O	88	91	1.0	1.0	

The solubility of acetic acid and phenol in solutions of different concentrations in collodion is shown in Table VI. The solubility of acetic acid is proportional to the concentration up to about molar, but above that the concentration of the acetic acid in the collodion increases more slowly than in the solution. The collodion begins to soften at about this concentration of acetic acid and can probably no longer be considered as pure collodion.

Since acetic acid is soluble in collodion while sodium acetate is not, it may be expected that the amount of acetic acid dissolved from a mixture of acetic acid and sodium acetate having the same total acetate concentration would be proportional to the amount of undissociated acetic acid in the mixture. Table VII shows that this is the case.

*Independent Solubility of Water and Acetic Acid, etc.*

According to the theory of ideal dilute solutions the quantity of substance dissolved should be the same irrespective of the presence of

TABLE VIII.  
*Weight and Composition of Solution Taken Up.*

Solution	Acetic acid		HgCl <sub>2</sub>	Phenol
	1 M	5 M	10 M	2 M
Weight collodion dry gm	422	422	434	287
“ “ + H <sub>2</sub> O	440	440	451	298
“ “ + solute	452	480	4542	306
Gm solute in collodion	012	040	0032	008
∞ moles solute × 10 <sup>6</sup>	20	66	1 2	8 5
Moles per gm. collodion × 10 <sup>6</sup>	47 5	156	2 7	28
Found by direct titration × 10 <sup>6</sup>	48	170	2 5	26
Moles per gm. collodion assuming solution of same concentration taken up by collodion.	7	14	46	1 3

Assume adsorption and that each mole HA displaces 1 mole H<sub>2</sub>O

Then total weight per gm. = 170 moles × 10<sup>-5</sup> HA + (240-170) × 10<sup>-5</sup> moles H<sub>2</sub>O

= .1 gm. HA + .012 gm. H<sub>2</sub>O

= .112 gm. total

Found .137

other solutes. The amount of water and of various solutes taken up by dry collodion is shown in Table VIII. The results agree with the assumption that the water and solute dissolve independently and cannot be accounted for by supposing that the membrane simply absorbs solution of the same concentration as the bulk of the solution. If it is assumed that the acid is adsorbed in the pores and that each mole of acid displaces one mole of water, the calculation also fails to agree with the experiment. It would be necessary from this point of view



to suppose that each mole of acid displaced much less than one mole of water, or else that the volume of the collodion increased.

*Effect of Sodium Chloride on the Permeability to Acetic Acid.*

If the acid diffuses through pores in the membrane it might be expected that the presence of concentrated salt solution on the opposite side of the membrane would increase the amount of acid passing through, since the salt forces water to pass through the membrane owing to osmotic pressure and some acid would be carried with the water. On the other hand, the presence of salt in solution with the acid would cause a stream of water to flow toward the acid and might be expected to decrease the amount passing through the pores. There are, however, possible secondary electrical effects or blocks due to partial closing of the pores by salt molecules, so that it is difficult to predict exactly the result of the experiment from this point of view. From the point of view of solubility the presence of the salt on the outside of the membrane should have a negligible effect since the concentration of acid is here practically 0. The presence of salt on the inside should increase the amount of acid passing through, since strong salt solutions increase the activity (vapor pressure) of the acid and should therefore increase the partition coefficient, and hence the permeability. The results of the experiment given in Table IX show that this latter prediction is qualitatively fulfilled but that quantitatively the increased solubility of the acid in the collodion due to the salt is not sufficient to account for the increase in permeability.

*Temperature Coefficient.*

If the substance diffuses through pores in the membrane the temperature coefficient of permeability should be that found in ordinary diffusion, unless it be further assumed that the pore area of the membrane changes with temperature. If passage takes place by solution almost any temperature coefficient might be expected since the temperature coefficients of solubility are irregular and may even be negative. The temperature coefficient of the *diffusion coefficient* in collodion should be small, however, unless some change occurs in the viscosity of the collodion. Table X gives the results of an experiment in which the rate of penetration of acetic acid was determined at 5°, 25° and 35°C. The

TABLE IX.  
*Effect of NaCl on Permeability of Acetic Acid.*

Inside Outside Permeability HA $\text{cm}^3/\text{day} \times 10^4$ Diffusion HA in collodion $\text{cm}^3/\text{day} \times 10^4$ Partition coefficient HA in collodion from	m/10 HA H <sub>2</sub> O	m/10 HA 5 m NaCl	m/10 HA + 5 m NaCl H <sub>2</sub> O	m/10 HA 5 m NaCl
	5 m NaCl solution = 1.2 1 m HA			
	028	029	042	027
	028	028	035	027

coefficient is about 2 for permeability over the whole range, and 1.6 for the diffusion coefficient from 5 to 25, but is the same as that for permeability from 25 to 35.

*Permeability to Water.*

Membranes made by placing in water before evaporation of the organic solvents may contain more than 5 times as much water as collodion but this water is not in equilibrium since it cannot be replaced once it has been removed without subjecting the membrane to some organic solvent. There is good reason to suppose that this water is held in the pores of the membrane and in fact that the pores are kept

TABLE X.  
*Permeability of 0.10 Molar Acetic Acid at Various Temperatures.*

Temperature	5°		25°		35°
$P = \text{cm}^3/\text{day} \times 10^4$	0 0086		0 038		0 085
$Q_{10}$		2 1		2 2	
$S = \frac{\text{Acid per cc. collodion}}{\text{Acid per cc. H}_2\text{O}}$	53		90		94
$D \times 10^4 = \frac{P}{S} =$	016		042		090
$Q_{10}$		1 6		2 1	

open by the surface tension effects of this water, as Zsigmondy (13) has suggested.

If such membranes are suspended over mixtures of sulfuric acid and water, or even over pure water, they lose weight and finally come to an equilibrium value with respect to water content. This small amount of water is in equilibrium with the membrane and may be removed and replaced repeatedly. The loss of water over pure water is presumably due to the pressure in the collodion network which causes the water to be forced out in droplets. These droplets have a convex surface and hence a higher vapor pressure than water in bulk. This is a slow process, however, and the loss of weight continues for months. The water content would presumably reach the value obtained by placing dry membranes in water.

The result of such an experiment is shown in Fig. 3 in which the grams of water per gram of collodion are plotted against the vapor pressure of water in the sulfuric acid-water mixtures. These figures are perfectly reversible and are obtained with either the dry membrane or the membrane made by immersing in water before the loss of all the organic solvent. There is some indication, however, that the equilibrium water content of the membranes increases with the original water content. This experiment seems to furnish evidence that water

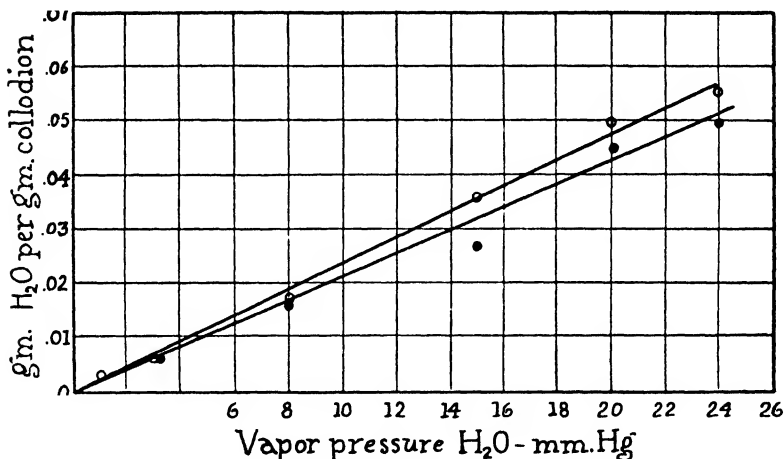


FIG. 3. Water content of collodion membranes over H<sub>2</sub>SO<sub>4</sub> water mixtures.

○ original water content of membrane 2.70 gm. H<sub>2</sub>O per gm. collodion.

● " " " " " 2.29 " " " " "

may be held in the membrane in two ways. First, as water held in the capillaries. This may be very large in amount and cannot be replaced once it is removed. Second, a small amount of water is dissolved in the collodion and the amount is proportional to the vapor pressure of water to which the membrane is exposed.

The result may, however, be explained qualitatively from the point of view of pores by assuming that the pores contract until the walls are in contact in places and that the residual water then in the membrane has a different vapor pressure depending on the size of the pore in which

it is held, since it is known that the vapor pressure of a liquid which wets a capillary decreases as the radius of the capillary decreases. From this point of view it is possible then to calculate the radius of these residual pores. The result of this calculation is shown in Table XI. It is evident that the calculated size of the pores is too large, since more than half the water is found to be in pores greater than  $1 \times 10^{-7}$  cm. radius or nearly 100 times the cross-sectional area of the molecules of gas which, from the permeability experiments with gases, are unable to pass through them. Also there is no reason to expect, from this point of view, that the amount of water in the pores should be proportional

TABLE XI.

*Radius of Pores from Vapor Pressure (14).*

$$\ln \frac{P_0}{P} = \frac{2 \sigma M}{RT \delta n}$$

$\sigma$  = surface tension = 72 ergs/cm.<sup>2</sup>

$M$  = molecular weight = 18 gm. mole<sup>-1</sup>

$\delta$  = density liquid = 1 gm./cm.<sup>3</sup>

$RT$  =  $2470 \times 10^7$  ergs mole<sup>-1</sup>

$r$  = radius capillary cm.

$P_0$  = vapor pressure liquid in bulk

$P$  = " " " " capillary

$P_0 = 24$

$P =$             1 2    3        8    15 20

$r \times 10^8$ , cm.    3 5    5 0    9 5    22 56

Water in

membrane

as per cent

of maximum    6    10    33    64 90

to the vapor pressure of the water. The calculation, however, includes the doubtful assumption that the angle of contact of the water and collodion is 0. There is also some question as to whether the equation can be applied to such small pores (18).

The permeability of the membranes to water may be determined by filling the membranes with salt or sugar solutions, immersing them in water and determining the increase in weight. Assuming that the water content of the membrane is proportional to the vapor pressure of the solution, the rate of diffusion in the collodion may be calculated. The result of such a calculation is given in Table XII. The vapor

pressure of the solution was calculated from the freezing point depressions in the case of the salt (neglecting the effect of temperature) and assumed proportional to the mole fraction of water in the case of the sugar solutions. It may be noted that the results with the salts although consistent for the various concentrations of the same salt are lower with  $\text{CaCl}_2$  than with  $\text{KCl}$  and sugar. This is the reverse of the result obtained by Lucke and McCutcheon (15) with sea urchin eggs.

If the water is assumed to flow through pores then the diameter of the pores may also be calculated from the above data, as was done by Hitchcock (5) and by Bjerrum and Manegold (6).

This calculation is shown in Table XIII. The size obtained in this way is of the order of magnitude of  $10^{-9}$ , or about 100 times smaller

TABLE XII.  
*Permeability of Membrane to Water When Filled with Various Solutions.*

Solution	Sugar		KCl		$\text{CaCl}_2$		
	10 M	0.50 M	1.0 M	0.5 M	1.0 M	0.5 M	0.25 M
Cc/day/unit membrane $\times 10^6$	1.25	53	1.2	60	1.45	74	27
Gm. $\text{H}_2\text{O}$ per cc. collodion water side—gm. $\text{H}_2\text{O}$ per cc. collodion solution side	00125	00062	00218	0011	0033	00175	00087
$D = \text{cm}^2/\text{day} \times 10^4$	100	85	55	55	44	42	31

than from the vapor pressure measurements. It also disagrees with the results of the gas measurements, since the radius is now smaller than the smallest molecular radius. There is again, however, some uncertainty as to the application of the equation to such small pores.

#### *Electroendosmosis.*

It might be supposed that the presence of pores could be tested for by electroendosmosis experiments. Since the effect of an electric potential is essentially the same as hydrostatic pressure, it should cause water to pass by diffusion also, and the experiment is therefore inconclusive.

*Permeability to Electrolytes.*

It has been found by Michaelis and his coworkers that dry collodion membranes act as reversible electrodes for cations but not for anions. That is, in low concentrations of salts a potential approaching the theoretical value of 55 millivolts is obtained when the membrane is placed between 0.10 and 0.01 molar KCl or other univalent neutral salt. These results have been accounted for by Michaelis by the

TABLE XIII.

*Calculation of Pore Size and Number from Permeability of H<sub>2</sub>O.*

Solution	Sugar		KCl		CaCl <sub>2</sub>		
	10 M	05 M	10 M	05 M	10 M	05 M	025 M
Cc. per day per cm <sup>2</sup> per cm thick membrane $\times 10^6$	1 2	53	1 17	61	1 45	74	27
Osmotic pressure atmospheres	22 4	11 2	39 5	19 5	60 5	31 4	15 7
Cc per day per atmosphere per unit membrane $\times 10^6$	056	048	029	031	024	023	017
Radius pore $\times 10^9$ cm	2 8	2 6	2 0	2 1	1 8	1 8	1 5
No. of pores per cm. <sup>2</sup> $\times 10^{-18}$	1 8	2 0	3 4	3 2	4 2	4 3	5 9

 $r$  = radius in cm.

1 = thickness collodion in cm. = 1

 $\eta$  = viscosity of water = .01 $Q$  = cc. water per sec. per dyne $m$  = gm. collodion per cc. = 1.65 $w = \frac{\text{Wet weight}}{\text{Dry weight}} = 1.04$ 

$$r = 1 \sqrt{\frac{8 \eta Q}{m(w-1)}}$$

$$n = \frac{m^2(w-1)}{8\pi Q 1^2 \eta}$$

 $n$  = no. of pores per cm.<sup>2</sup>

assumption that the anions are prevented from entering the pores due to an electrical block. The cations can enter the pores but are held back by the attraction of the anions. When a salt solution is present on both sides of the membrane, therefore, the cations can pass through but the anions cannot, so that in the pores the mobility of the cations is much greater than that of the anions. Under these conditions the diffusion potential in the pores should be the same as that expected from a reversible electrode, and this is the case. It would be expected from this point of view that an exchange of cations should take place

when a salt solution is present on both sides of the membrane. Michaelis (16) has found this to be true, but the amount of cations passing through the membrane is exceedingly small. The membranes used in the present experiments gave a concentration potential when placed between 0.10 and 0.01 M KCl of 30 to 40 millivolts. They were impermeable to all electrolytes tried except  $\text{HgCl}_2$ . This salt is more soluble in alcohol than in water and has a measureable solubility in collodion, as might be expected. As noted in Table I it is fairly permeable. About one-third of the membranes tested showed measurable permeability to other electrolytes but the results were irregular and the majority showed no permeability even after a month. It seems necessary to assume that in such experiments the minimum figure is the correct one. The experiments in which the membrane was placed between two salt solutions were also irregular. In the majority of cases no exchange of cations could be noted while in those cases where an exchange occurred there was also some passage of anions. It is possible that this difference from Michaelis' results is due to the different collodion and method of making the membranes. In the case of sodium or potassium acetate separated from HCl, however, there is regularly a passage of the salt through the membrane while no passage occurs when pure water is on the other side of the membrane. The result of such an experiment is shown in Table XIV. It will be noted that both ions of the salt pass through. This result would be expected, however, if the cations could exchange as suggested by Michaelis, since in this case the acetic acid formed can penetrate. If potassium ions passed through, therefore, in exchange with hydrogen ions, acetic acid would be formed in the acetate solution and since the acetic acid could penetrate it would in turn diffuse through and this would continue until the concentration (activity) of the undissociated acetic acid became equal on the two sides of the membrane. The net result would be that both potassium and acetate ions pass through the membrane. From the point of view of solubility these results can be accounted for by assuming that the collodion contains a small amount of an organic acid in solution whose salts are also soluble in collodion. (16). The membrane would therefore act as a reversible electrode for cations as soon as an amount of cation equivalent to the organic acid was taken up since the concentration of the cation in the membrane



would now be constant, a necessary and sufficient condition for a reversible electrode. The low potentials found by Michaelis between concentrated salt solutions would on this basis be due to a very slight independent solubility of the salt itself in the membrane. This explanation has an advantage in that it predicts that the exchange of cations would be very small. As shown in Table XIV this is the case, the penetration of potassium or acetate ion being about 1/10 that for acetic acid. If the impermeability to these ions were due to an electrical block which prevented them from entering the pores, it would be expected when the block was removed by arranging the experiment so that an exchange of ions could take place that the rate of passage of the ions would be of the same order of magnitude as that of molecules of the same size.

TABLE XIV.  
*Permeability of Potassium Acetate, 30 Days.*

Inside	0 10 M. KA		0 10 M KA	
Outside	1 M HCl		H <sub>2</sub> O	
Moles Cl <sup>-</sup> inside × 10 <sup>6</sup>	2 0	0		
Moles outside × 10 <sup>6</sup>	K 6 6	4 0	Trace	Trace
	Acetate 6 0	5 0	"	"
Membrane factor	2 × 10 <sup>-8</sup>	2 × 10 <sup>-8</sup>	2 × 10 <sup>-8</sup>	2 × 10 <sup>-8</sup>
Permeability × 10 <sup>7</sup>	Cl 1 0	0		
	K 3 3	2 0	0	0
	A 3 0	2 5	0	0

It is also possible that the membranes contain a few small pores having the properties assumed by Michaelis. The potential developed is independent of the number of pores so that the electrical properties of the membranes may be determined by these occasional and perhaps accidental openings, whereas the actual permeability is determined by solubility and diffusion since if the total pore area is minute the amount of substance passing through them would be negligible.

An essential difference between the two mechanisms exists in the prediction of the results with electrolytes. If the permeability depends on pores and the electrolytes are prevented from passing by an electrical block, then no strong electrolyte can pass. If, however, the permeability depends on solution in the collodion it is quite possible that some electrolytes would have this property and the membrane

would therefore be permeable to them. Mercuric chloride, however, can hardly be cited as an example of this prediction since it is only slightly ionized.

#### SUMMARY.

The rate of penetration and the solubility of H, O, N,  $\text{NH}_3$ ,  $\text{H}_2\text{O}$ , HCl gas,  $\text{CO}_2$ , formic, acetic, chloracetic, dichloracetic acid, glycerol, phenol and mercury bichloride in dry collodion membranes have been measured.

The rate of penetration of H and  $\text{CO}_2$  is the same whether the membrane and gas are dry or whether the membrane is immersed in water.

The solubility of  $\text{CO}_2$ , acetic acid, phenol and water in collodion is completely reversible and is proportional to the concentration (or vapor pressure) in low concentrations and independent of the surface of the collodion.

The size of the pores has been calculated from the vapor pressure of water in the collodion and from the rate of flow of water through the membrane. The results do not agree and are not consistent with the observed rates of penetration.

The relative rates of penetration of the gases bear no relation to the density of the gas.

When the results are corrected for the solubility of the substances in the collodion and expressed as the diffusion coefficient in collodion they show that the diffusion coefficient increases rapidly as the molecular weight decreases.

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## SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

### V. RESEMBLANCES BETWEEN THE LIVING CELL AND AN ARTIFICIAL SYSTEM IN ABSORBING METHYLENE BLUE AND TRIMETHYL THIONINE.

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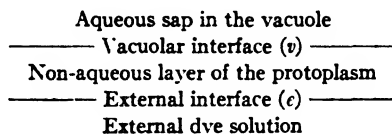
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#### I.

#### INTRODUCTION.

This paper deals with an attempt to imitate the action of the living cell in respect to penetration of dyes.

The protoplasm of a living cell probably consists of two non-aqueous layers<sup>1</sup> (one in contact with the external medium and the other with the vacuolar sap), which are separated by an aqueous middle layer. But in setting up an artificial system we may confine ourselves to the simple case where the rate of diffusion appears to be controlled by three phases, as if a living cell consisted of only one non-aqueous layer lying between the external solution and the aqueous sap of the vacuole, as shown by the following diagram:



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<sup>1</sup> We are unable to say which layer plays the more important rôle in regulating the rate of penetration into the vacuole. The controlling factor may change with alterations in the condition of the cells brought about by experiments. It seems possible that the external non-aqueous layer is more polar than the vacuolar layer, in that the ions penetrate the former more rapidly than the latter. In the case cited in the text the vacuolar non-aqueous layer may be responsible for the control of the rate. But in any case it seems probable that the net result may be treated as if the rate were controlled by a system containing only one non-aqueous layer.

In such a system the diffusion of a dye will be greatly affected by the partition coefficients:

$$K_e = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the external solution}}$$

$$K_s = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the aqueous sap}}$$

The higher  $K_e$  is, the more rapid is the rate of diffusion from the external solution through non-aqueous layer. On the other hand, the lower  $K_s$  is, the more rapid is the rate of diffusion through the non-aqueous layer. The values of  $K_e$  and  $K_s$  and concentrations of dye in the aqueous solutions will determine the diffusion gradient in the non-aqueous layer.

In the case of methylene blue, diffusion into the vacuole from the external solution is very slow because  $K_e$  is so small that though  $K_s$  is low there is very little dye in the non-aqueous layer<sup>2</sup> to diffuse into the sap. When a dye exists in two forms, with free base predominating at higher pH values and salt predominating at lower pH values, it is necessary to consider the partition coefficient of each form of dye at each interface. In the case of azure B the partition coefficient of the dye in the form of free base,<sup>3</sup>  $K_{eb}$ , is so high that its diffusion through

<sup>2</sup> According to Overton's theory penetration depends on the solubility of the dye in the lipid layer of the living cell, which involves only one partition coefficient,  $K_e$ . This is sufficient to account for the lack of penetration of dyes which are insoluble in lipid and for the rapid penetration of some of the dyes soluble in lipid, but does not explain why a dye like crystal violet, which is very soluble in lipid, does not readily penetrate the cell. On the basis of the theory presented in the text the slow penetration of crystal violet into the cell is explainable; though the dye penetrates the non-aqueous layer it does not enter the vacuole rapidly on account of  $K_s$  being so high.

<sup>3</sup> Though the dye in form of free base is often considered to be undissociated, this is an open question. The free base may be dissociated just as much as the dye in form of salt. We must therefore leave the question of the extent of dissociation of the "free base" for future solution by organic chemists, while we may conclude with more certainty that the "salt" is dissociated. This "free base" must be distinguished from the "pseudo base" which exists at still higher pH values. "Free base" may possibly be represented by an anhydro-base, or by a structure like the salt except that the halide is replaced by the hydroxyl group. (Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 927 and 928, foot-note 3.)

non-aqueous layer from the external solution is rapid. But even if  $K_{\text{v}}$  (the partition coefficient of the same form of dye at the vacuolar interface) is high, the dye readily diffuses through the non-aqueous layer into the vacuole because it is transformed to salt as soon as it comes in contact with the sap (on account of the low pH value of the sap) and this greatly increases the diffusion gradient. Since the partition coefficient of the dye in the form of salt at the vacuolar interface,  $K_{\text{v}}$ , is low, its backward diffusion from the vacuole through non-aqueous layer is very slow. The dye salt readily collects in the vacuole. The same type of behavior occurs if a dye enters into combination with the sap to form a very slightly soluble compound.

This view would explain why azure B penetrates more rapidly than methylene blue into the vacuole of a living cell, as described in previous papers,<sup>4-6</sup> and would lead us to predict that the same thing would happen with an artificial system consisting of (1) the same dye solutions as those employed for the living cells, (2) chloroform to represent the non-aqueous layer of the protoplasm, and (3) sap freshly extracted from the vacuoles of living cells or artificial sap. Since the non-aqueous part of the protoplasm is but crudely represented by chloroform, the system may bear only a qualitative resemblance to the living cell.

If this theory<sup>7</sup> be correct we shall expect that when mixtures of dyes are employed the order of penetration will be the same in the artificial system as in the living cell.

## II.

### *Methylene Blue.*

#### *A. Comparison of the Artificial System with Valonia.*

Previous analyses<sup>4,6</sup> have shown that the vacuole of *Valonia* takes up azure B from a solution of methylene blue in sea water at pH 9.5. In order to compare this with the artificial system two types of

<sup>4</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 425.

<sup>5</sup> Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 927.

<sup>6</sup> Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147.

<sup>7</sup> This theory has been successfully tested in the case of many dyes (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127; 1928, xxvi, 125).

experiments were made. (1) One experiment is to show the absorptive power of the chloroform in relation to the aqueous dye solution (representing the external dye solution used for living cells). This is done by first shaking the chloroform with the aqueous dye solution, allowing the chloroform to evaporate, and then dissolving the dye residue in the sap or in distilled water. The dye thus obtained is called for convenience "the dye obtained from chloroform by evaporation."

(2) The second experiment is to determine how readily the chloroform is able to give up the dye to the sap. This is done by first shaking the chloroform with the aqueous dye solution and subsequently extracting the dye from the chloroform by shaking it up with the sap.

If chloroform adequately represents the non-aqueous layer of a living cell we may by studying the behavior of the chloroform<sup>8</sup> toward the dye in these two aqueous phases learn something of the behavior of the non-aqueous layer of the cell in relation to phases inside and outside the cell.

The experiments were therefore carried out as follows.

200 cc. of solution of methylene blue (French) dissolved in sea water at pH 9.5 or at pH 5.5 was shaken up with 50 cc. of pure chloroform in a separatory funnel; the chloroform was then removed from the funnel and if it contained even a trace of water in the form of droplets the latter was removed by decanting the chloroform repeatedly from one beaker to another: the drops of water then adhered to the wall so that eventually chloroform free from drops of water could be obtained by this method. The chloroform was then divided into two equal parts: one portion was allowed to evaporate and the dye residue was dissolved in freshly extracted sap of *Valonia* (the sap contained about 0.6 M halides and had a pH value of about 5.8) or in distilled water. The second portion was shaken with sap until a concentration sufficient for spectrophotometric analysis was obtained. The sap was separated from the chloroform by pipetting it off (there was not sufficient chloroform in the sap to affect the absorption spectra of the dye in the sap).

The sample of methylene blue (French) gave an absorption curve with primary absorption maximum at 664 m $\mu$  when dissolved in sea water at pH 9.5 or pH 5.5 (Fig. 1, symbol  $\square$ ), which is characteristic of a dye solution consisting chiefly of methylene blue.

<sup>8</sup> Though the non-aqueous layer is represented by the chloroform, it does not signify that it resembles chloroform in chemical composition. It is uncertain as to whether the non-aqueous layers are "lipoid," but their behavior suggests it. The question of the character of the non-aqueous layers must therefore be left undecided for the present.



With this sample of methylene blue in sea water at pH 9.5, the dye (1) obtained from chloroform by evaporation, and (2) extracted from the chloroform by shaking with sap, gave an absorption curve characteristic of a dye consisting chiefly of azure B (with primary absorption

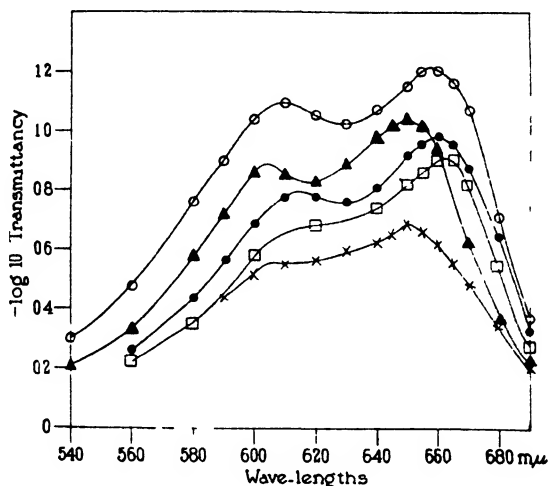


FIG. 1. Absorption curves obtained in experiments on methylene blue (French). (Comparison with *Valonia*.) Symbol  $\square$  represents the methylene blue dissolved in sea water at pH 5.5 or pH 9.5. Symbol  $\blacktriangle$  represents the dye taken up by chloroform from methylene blue in sea water at pH 9.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol  $\times$  represents the dye extracted by sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 9.5. Symbol  $\circ$  represents the dye taken up by chloroform from methylene blue in sea water at pH 5.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol  $\bullet$  represents the dye extracted by the sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 5.5. The measurements were made on a layer 1 cm. thick.

maximum at 650  $m\mu$ ; Fig. 1, symbols  $\blacktriangle$  and  $\times$ ), which readily enters the chloroform because at pH 9.5 it is largely in the form of free base; it readily comes out into sap because the acidity of the sap changes it to dye salt which has a low value of  $K_s$ . The reddish violet of the dye in chloroform may indicate the absorption chiefly of azure B in the

form of free base and a smaller amount of azure B in form of salt and methylene blue. With the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 658  $m\mu$  (Fig. 1, symbol  $\circ$ ) and the dye extracted by the sap from the chloroform gave a primary absorption maximum at 660  $m\mu$  (Fig. 1, symbol  $\bullet$ ), indicating that in both cases there was a mixture of azure B and methylene blue (the former containing slightly less methylene blue than the latter). The color of the dye in chloroform was blue, thus showing that if azure B was absorbed it was in form of salt. The amount of azure B in form of salt and of methylene blue absorbed is less than in the case of azure B in form of free base.

The result obtained at pH 9.5 is in complete agreement with that obtained with living cells of *Valonia* (uninjured). Unfortunately it is not possible to compare the result with that of the living cell in solutions at pH 5.5 since in the latter case penetration is so slow that analysis is not possible at present. But if the present theory be correct we might expect a mixture of these two dyes at pH 5.5 to penetrate the cell but only more slowly than azure B from the solution at pH 9.5.

#### *B. Comparison of Nitella flexilis with the Artificial System.*

Previous experiments<sup>9</sup> have shown that from methylene blue solution (French or Merck's medicinal) at pH 9.2 azure B is readily taken up by the vacuole of living cells of *Nitella*. For comparison with the behavior of an artificial system the following experiments were carried out.

The sample of methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 or pH 5.5 gave an absorption curve characteristic of methylene blue (Fig. 2, symbol  $\blacksquare$ ).

The same technique as described under *A* was employed (1) for absorption of dye by chloroform from this sample of methylene blue dissolved in diluted buffer solution at pH 9.2 and pH 5.5, and (2) for the extraction of dye from the chloroform by the artificial sap of *Nitella*. It was not possible to use the real sap

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<sup>9</sup> In 1926-27 preliminary statements of these results on *Nitella* were made on page 426 of the paper referred to in foot-note 4, and on page 945 of the paper referred to in foot-note 5. In 1928 these results in detail were reported in the paper referred to in foot-note 6, and in *Proc. Soc. Exp. Biol. and Med.*, 1927, **xxv**, 563.

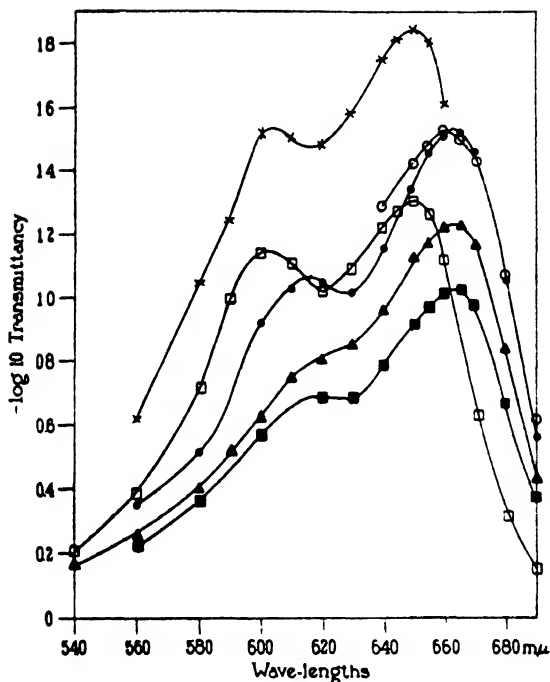


FIG. 2. Absorption curves obtained in experiments on methylene blue solution (Merck's medicinal). (Comparison with *Nitella*.) Symbol ■ represents methylene blue solution at pH 9.2 or at pH 5.5. Symbol □ represents the dye absorbed by chloroform from methylene blue solution at pH 9.2, freed from chloroform by evaporation, and then dissolved in artificial sap of *Nitella*. Symbol × represents the dye extracted by artificial sap of *Nitella* from chloroform which has absorbed the dye from methylene blue solution at pH 9.2. Symbol ○ represents the dye absorbed by chloroform from methylene blue solution at pH 5.5. The chloroform was allowed to evaporate and the dye residue was dissolved in artificial sap of *Nitella*. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform previously shaken with methylene blue solution at pH 5.5. Symbol ▲ represents the dye extracted with distilled water at pH 5.5 from chloroform previously shaken with methylene blue solution at pH 5.5. The curves with the symbols ●, ■, × refer to measurements made with a layer 1 cm. thick, the others to measurements made with a layer 0.3 cm. thick.

of *Nitella* because the protein coagulates on shaking. The artificial sap was therefore made up with 0.11 M KCl dissolved in a solution of M/150 phosphate buffer at pH 5.5. All measurements described in the text were made immediately after extractions.

With the solution at pH 9.2 the dye obtained from chloroform by evaporation or by extraction with the sap gave an absorption curve characteristic of a dye containing chiefly azure B with a primary ab-

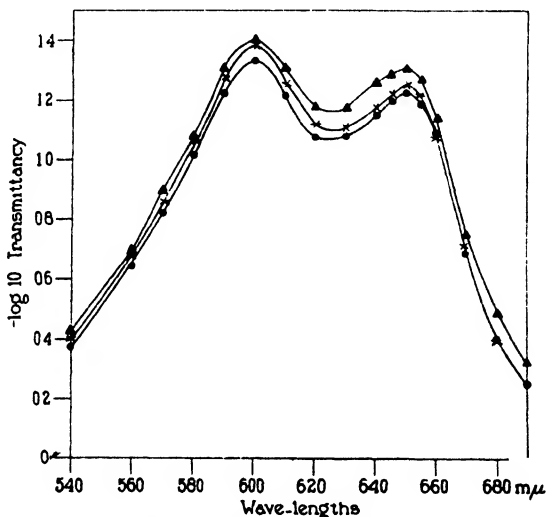


FIG. 3. Absorption curves obtained in experiments on azure B or trimethyl thionine (Holmes) in sea water. (Comparison with *Valonia*.) Symbol  $\times$  represents the azure B dissolved in sea water at pH 9.5 or pH 5.5. Symbol  $\bullet$  represents the dye extracted by sap of *Valonia* from chloroform shaken with azure B in sea water at pH 9.5; symbol  $\blacktriangle$  the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

sorption maximum at 650 mμ (Fig. 2, symbol  $\square$  and  $\times$ ). The color of the dye in chloroform was violet red thus indicating that azure B in the form of free base was absorbed. But with the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 660 mμ (Fig. 2, symbol  $\circ$ ); the dye extracted by the sap from the chloroform gave a primary absorption maximum at 663 mμ (Fig. 2, symbol  $\bullet$ ), indicating that a mixture containing chiefly

methylene blue with some azure B was absorbed by the chloroform, but mostly methylene blue was extracted from the chloroform by the sap. The color of the dye in chloroform was blue, thus showing that some azure B in form of salt or methylene blue was absorbed.

When the experiments were repeated at pH 9.2 with methylene blue (French) the same result was obtained as with Merck's medicinal.

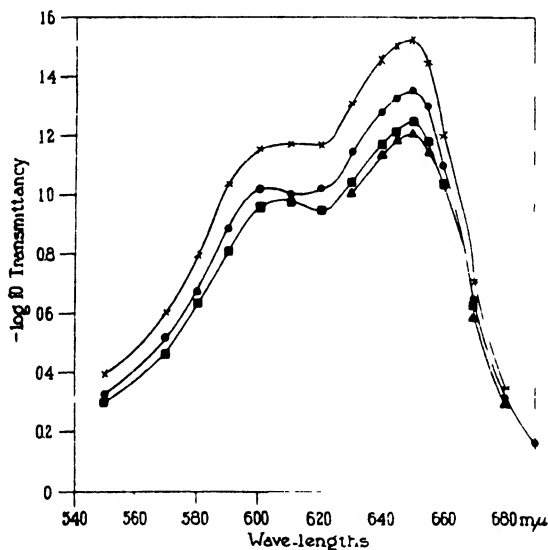


FIG. 4. Absorption curves obtained from experiments on azure B (Holmes) dissolved in buffer solutions. (Comparison with *Nitella*.) Symbol ■ represents the dye dissolved in buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye absorbed by chloroform from azure B solution at pH 9.2. The dye was freed from chloroform by evaporation and dissolved in distilled water. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform shaken with azure B solution at pH 9.2; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

The results obtained with methylene blue at a pH value a little above 9 were in general agreement with the experiments on living cells (uninjured). At pH 5.5, the comparison is not possible since the penetration is so slow that there is not sufficient dye collected for analysis in the sap before there was a possibility of injury to the cell.

## III.

*Azure B or Trimethyl Thionine.*

Using the method described in section II experiments were repeated with azure B for comparison of the artificial system with *Valonia* (Fig. 3) and with *Nitella* (Fig. 4). In both cases the dye absorbed by chloroform and set free by evaporation or extracted by the sap from chloroform gave the absorption curve of a dye consisting chiefly of azure B with a primary absorption maximum at  $650\text{ m}\mu$  (Fig. 3, symbols  $\bullet$  and  $\blacktriangle$  and Fig. 4, symbols  $\times$ ,  $\bullet$ , and  $\blacktriangle$ ), which is identical with the dye solutions in which chloroform was shaken up (Fig. 3, symbol  $\times$  and Fig. 4, symbol  $\blacksquare$ ). These results are in exact agreement with those obtained with uninjured cells of *Valonia* and of *Nitella*.

## IV.

## CONCLUSION.

The rate of diffusion<sup>10</sup> through the non-aqueous layer of the protoplasm depends largely on the partition coefficients mentioned above. Since these cannot be determined we have employed an artificial system in which chloroform is used in place of the non-aqueous layer of the protoplasm. The partition coefficients may be roughly determined by shaking up the aqueous solutions with chloroform and analyzing with the spectrophotometer (which is necessary with methylene blue because we are dealing with mixtures). This will show what dyes may be expected to pass through the protoplasm into the vacuole in case it behaves like the artificial system.

From these results we may conclude that the artificial system and the living cell act almost alike toward methylene blue<sup>11</sup> and azure B, which supports the notion of non-aqueous layers in the protoplasm.

There is a close resemblance between *Valonia* and the artificial system in their behavior toward these dyes at pH 9.5.

<sup>10</sup> Cf. Northrop, J. H., *J. Gen. Physiol.*, 1928-29, xii, 435.

<sup>11</sup> Methylene blue solution is generally found to contain azure B as impurity but in too small a concentration to affect the primary absorption maximum which is at about  $665\text{ m}\mu$ . It is, however, possible to determine roughly by extraction with chloroform the relative amount of azure B present in methylene blue solutions

In the case of *Nitella*, on the other hand, with methylene blue solution at pH 9.2 the sap in the artificial system takes up relatively more azure B (absorption maximum at 650  $m\mu$ ) than the vacuole of the living cell (655  $m\mu$ ). But both take up azure B much more rapidly than methylene blue.

A comparison cannot be made between the behavior of the artificial system and that of the living cell at pH 5.5 since in the latter case there arises a question of injury to cells before enough dye is collected in the sap for analysis.

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since azure B in form of free base is absorbed by chloroform from methylene blue solution much more rapidly than methylene blue itself. Azure B may therefore be readily extracted from methylene blue solution at about pH 9. The dye thus extracted is freed from chloroform by evaporation and is dissolved in various aqueous solutions. The dye thus extracted contains various proportions of azure B and methylene blue giving primary absorption maxima from 650  $m\mu$  (that of the pure azure B) to 662  $m\mu$  (nearly that of purest available methylene blue), depending on the amount of azure B in proportion to methylene blue present in the methylene blue solution from which the extraction was made.

Another method of detection is by observing the color of the dye in chloroform since azure B in form of free base appears violet red, while methylene blue appears blue. But this method is inadequate if the extracted dye is a mixture or if the concentration is low where color matching becomes difficult.

At about pH 9 we find that the more azure B a methylene blue solution contains the more rapid is the rate of penetration of the blue dye into the vacuole of a living cell as well as into the artificial "vacuole."

If the penetration is very slow there are possibilities of errors arising from injury and contamination as already stated (Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147) so that the sample "French" was chosen, which had a higher rate of penetration than any other samples (it contained more azure B than others). With *Valonia*, even with the sample "French," the rate of penetration was so slow that it could not very well have been lowered without rendering the results doubtful on account of the possibilities of error already stated. For this reason conclusive results were not possible from experiments with other samples which showed a slower rate of penetration. But with *Nitella* the penetration was more rapid with each of the samples employed so that it was possible to employ a greater variety of samples.

The analogy between chloroform and the living cell is only a rough one. The resemblance, for example, exists in so far as both systems (living and artificial) take up azure B much more rapidly than methylene blue but we need not suppose that the dyes absorbed by both systems will give exactly identical absorption curves in every instance.

These resemblances both in *Valonia* and in *Nitella* become less as injury increases on account of the increase in penetration of methylene blue into the vacuoles of injured cells.



## THE EFFECT OF DIFFUSION AT A MOVING BOUNDARY BETWEEN TWO SOLUTIONS OF ELECTROLYTES.

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(Communicated November 27, 1928.)

In the determination of the transference number of an electrolyte by the method of moving boundaries, a sharp boundary is formed between the solution to be studied (an aqueous solution of silver nitrate, for instance) and another "indicator" solution containing an electrolyte with a common ion and an ion of lower mobility. Lithium nitrate fulfils these conditions as an indicator for silver nitrate. Such a boundary will move when a current is passed through it. From measurements on this motion the transference number  $T$  of one ion constituent of the leading solution may be computed from the formula

$$T = \frac{v}{V} \cdot \frac{F}{it} \quad (1)$$

in which  $v$  is the volume swept through by the boundary,  $V$  the volume containing 1 gram equivalent of the electrolyte,  $F$  the faraday equivalent,  $i$  the current, and  $t$  the time. It has, however, been found necessary to have the concentration of the indicator solution adjusted so as not to be very far from the concentration given by the relation

$$C' = C \frac{T'}{T}$$

in which  $C$  and  $C'$  are the concentrations of the leading and indicator solutions and  $T$  and  $T'$  the corresponding transference numbers. In recent years it has been shown that the method of moving boundaries is capable of high precision.

Since the plane of contact between solutions of two electrolytes is not, under usual conditions, either permanent or definite, it became of interest to see whether the movement of the boundary is affected by

diffusion of one electrolyte into the solution of the other, as, for instance, the diffusion of silver nitrate into the lithium nitrate solution, and vice versa. To obtain data upon the effect of such a diffusion the experiment was made of stopping the motion of the boundary, by stopping the current, during the progress of a determination of the transference number of 0.1 *N* silver nitrate. Our usual arrangement for the determination of the transference number by the moving boundary method was used, with the cell,<sup>1,2</sup> constant current regulator,<sup>3</sup>

TABLE 1.

*Determination of the Transference Number of 0.1 N Silver Nitrate at 25° by the Moving Boundary Method. Experiment with Interruptions.*

Reading	Volume	Time	Velocity of boundary × 10 <sup>4</sup>	Transference number
cm.	cc.	seconds	cc per sec.	
1 0	0 1630	484	3368	0 4642
2 0	0 3284	965	3404	0 4691
3 0	0 4929	1449	3401	0 4689
Current interrupted 9 minutes				
4 0	0 6603	1938	3407	0 4696
5 0	0 8248	2432	3391	0 4675
Current interrupted 15 minutes				
6 0	0 9909	2919	3395	0 4679
7 0	1.1580	3406	3400	0 4686
Current interrupted 30 minutes				
8 0	1 3242	3900	3395	0 4680
9.0	1 4919	4391	3396	0 4683
10 0	1.6600	4890	3395	0 4679

and vibration-free support<sup>3</sup> already described. The indicator solution was 0.078 *N* lithium nitrate, found by previous experiment to be within the "adjustment range" for this determination. The experiment differed from the regular determination in that at three periods during the run the current (7 milliamperes) was cut off. The data taken during this run are given in table 1. The timing device giving the figures in the third column of the table operated only during the time that the current was on. Upon stopping the current the boundary, which had been very sharp and easy to locate, gradually faded out, and at the end of about a minute no evidence of discontinuity between the

two solutions could be observed. However, upon starting the current again the boundary slowly reformed, and, after a period depending upon the time the current was off, fully recovered its original sharpness.<sup>4</sup> As is shown in the table, interruptions of 9, 15, and 30 minutes were made during the run. Each interruption occurred at a graduation of the measuring tube. The boundary regained its original sharpness and was easily read at the next 1 cm. graduation, even after the half-hour interruption.

The most surprising observation from this experiment is, however, that the diffuse zone between the two solutions, which was for a time quite invisible and later only faintly visible as a boundary, moved at just the same rate as the fully formed boundary.

This is shown by the values of the velocity  $v/t$  given in column 4 of table 1. These velocities are constant within the limit of error. It is evident that the diffusion zone must have been moving at this constant velocity even when invisible. The transference numbers computed from Equation 1 are given in the last column. The value of this constant obtained by averaging the results of determinations obtained from measurements of 7 cm. or over is

0.4682 which agrees closely with the value 0.4685 obtained by numerous other measurements that we have made on this constant.

In order to obtain a more definite idea of the extent of the diffusion which took place when the current was interrupted, readings were made on a boundary consisting of 0.1 *N* potassium permanganate as leading solution and 0.065 *N* potassium acetate as indicator. The motion of the sharp junction between the colored and colorless anions was interrupted for 30 minutes at a graduation of the measuring tube when the run had proceeded for some time. At the moment of interruption the boundary had the appearance of figure 1(a). During the interruption the permanganate diffused up the tube for about 3 mm. giving the appearance of figure 1(b). The diffused or mixed zone between the two solutions must therefore have been about 6 mm. thick. On restarting the current this diffusion layer gradually narrowed and at the end of 10 minutes, during which time a normal boundary would have moved 1 cm., the junction had regained its

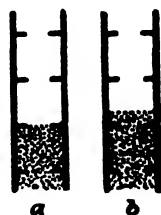


FIG. 1.

original sharpness. Here again the readings observed were exactly those which would have been obtained if the interruption had not occurred.

It is quite evident, therefore, that there is a powerful mechanism tending to restore a boundary to its original condition if diffusion takes place. The action of this mechanism is probably as follows. Due to the passage of current there is a drop of potential in both solutions. The potential gradient in the leading solution is, however, lower than in the following solution which contains ions of lower mobility and is also more dilute. The relations are shown diagrammatically in figure 2 in which potentials are ordinates and distances along the measuring

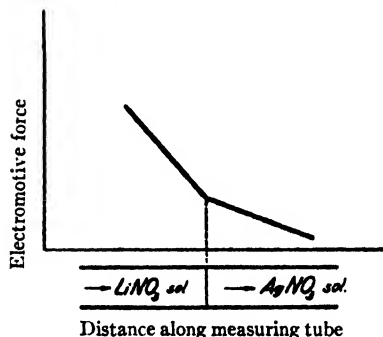


FIG. 2.

tube are abscissae. If, under these conditions, the relatively fast moving silver ions diffuse back into the lithium nitrate solution they encounter a high potential gradient and are rapidly sent forward to the boundary. On the other hand, if lithium ions diffuse into the silver nitrate region they move slower than the silver ions and will be finally overtaken by the moving boundary. If extensive diffusion is allowed to occur, as in the experi-

ments described above, there will not be a sharp break in the potential as shown in figure 2, but there will be, however, a higher gradient behind any given layer of solution in the diffuse zone than there is in front. Although the presence of this restoring mechanism must have been recognized by workers in this field the only published mention of it we have found is a brief note in a paper by Denison.<sup>5</sup> It seems probable that the visibility of the boundary is largely determined by the change of potential gradient at that plane. Thus the silver nitrate-lithium nitrate boundary is readily observed whereas if the lithium nitrate is replaced by the corresponding sodium salt the resulting boundary is followed with the greatest difficulty. The relatively slight difference in the refractive indices of the two indicator solutions does not seem to be sufficient to account for the large differ-

ence in visibility. The difference between the potential gradients in the leading and indicator solutions is, however, cut to less than half by the substitution of sodium for lithium in the latter solution.

<sup>1</sup> MacInnes, D. A., and Brighton, T. B., *J. Amer. Chem. Soc.*, **47**, 994, 1925.

<sup>2</sup> MacInnes, D. A., Cowperthwaite, I. A., and Huang, T. C., *J. Amer. Chem. Soc.*, **49**, 1710, 1927.

<sup>3</sup> MacInnes, D. A., Cowperthwaite, I. A., and Blanchard, K. C., *J. Amer. Chem. Soc.*, **48**, 1909, 1926.

<sup>4</sup> Steele, B. D., *J. Chem. Soc.*, **79**, 423, 1901, noticed the reformation of a boundary after interruption of the current, but, in his observation "never with such clearness as in an undisturbed experiment."

<sup>5</sup> Denison, R. B., *Z. physik. Chem.*, **44**, 583, 1903.



## ACTIVE AND PASSIVE IMMUNITY TO PNEUMOCOCCUS INFECTION INDUCED IN RABBITS BY IMMUNI- ZATION WITH R PNEUMOCOCCI.

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In a previous publication (1) the fact was established that rabbits, immunized with degraded, avirulent, non-type-specific pneumococci—so called R strains derived from any one of the three types—acquire a considerable degree of resistance against subsequent infection with virulent Type III pneumococci. Active resistance was demonstrable under these conditions in spite of the fact that the sera of the immunized rabbits contained no type-specific antibodies capable of agglutinating Type III S cells, or of precipitating the soluble specific substance derived from Type III cultures, or of conferring passive protection on mice against Type III infection. It was suggested (1) that this form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of pneumococcus used for immunization, differed in principle from type-specific immunity. The previous experiments were restricted to the study of the active resistance to *Pneumococcus* Type III. However, it seemed possible that this form of immunity—induced by immunization with R cells—might be effective against infection with other virulent types of pneumococcus. Consequently, additional studies have been carried on to determine whether the apparently non-type-specific resistance thus induced is as effective against Type I and Type II as against Type III. The results are recorded in this communication. In view of the differences which appear to exist between the form of active immunity stimulated in rabbits by type-specific S organisms and intimately associated with type-specific antibodies, and active resistance induced by prolonged immunization with R cells, further investigation into the nature and mechanism of the latter type of immunity is in progress.

In addition, the work has been extended to include passive immunity in order to determine whether the blood of rabbits immunized with R pneumococci, is capable of conferring protection upon normal animals of the same or of different species. The results of experiments on passive immunity are also included in this report.

### *Methods.*

*Antigens.*—An R strain originally derived from Type II S culture (designated R<sub>2</sub>), an R strain similarly derived from Type I S culture (designated R<sub>1</sub>), and a Type III S strain were employed for the immunization of all rabbits used in the experiments on active immunity. Only the R<sub>2</sub> strain was used for immunization in the tests for passive immunity.

Vaccines were prepared from 12 to 14 hour plain broth cultures. The organisms, removed from the broth by centrifugation, were resuspended in physiological salt solution in such proportion that 0.5 cc. of the suspension was equivalent in bacterial content to 1 cc. of original culture, and heat-killed at 56° for 30 minutes.

*Technique of Immunization.*—The method of immunization, described by Cole and Moore (2), consisted of a total of 18 intravenous injections; 0.5 cc. doses of vaccine were given daily for 6 days followed by a week of rest until the procedure was repeated 3 times. As a rule, the animals were tested 9 to 14 days after the last dose.

*Protection Tests.*—12 to 14 hour blood broth cultures of virulent pneumococci of Types I, II, and III were used in all tests for active and passive immunity. Type I cultures possessed a maximum virulence for rabbits of 0.000001 cc.; Type II were usually fatal in 0.000001 cc. and always in 0.00001 cc. amounts; Type III killed regularly at 0.0001 cc. In most of the experiments, in order to follow the degree of the bacteremia, blood cultures were taken at frequent intervals during the course of infection as previously described (3).

Rabbits tested for increased resistance to Type I infection had previously been treated as follows: 1 had been immunized with R pneumococci derived from Type I S strain, 15 with R pneumococci derived from Type II S strain, and 3 with Type III S pneumococci. Their sera were tested for the presence of agglutinins for R organisms and for the S forms of the three specific types. In no instance were type-specific agglutinins demonstrable; there were, however, agglutinins for R cells present in titres of 1:640 to 1:1280. Antibody response was similar regardless of organisms used for immunization. That rabbit avirulent strains of Type III stimulate in rabbits antibody formation of the same character as that elicited by R cells has been previously described (4). The infecting dose of Type I pneumococcus, in every instance, represented 10,000 to 100,000 lethal doses. By keeping the test strain at a maximum virulence for rabbits of 0.000001 cc., 0.01 cc., or 0.1 cc. of such culture constituted the dosage of organisms employed.



In each experiment, in addition to the immunized rabbits, normal animals receiving the minimal lethal dose and others the test dose served as controls of the virulence of the culture.

TABLE I.

*Active Immunity against Infection with Pneumococcus Type I in Rabbits Immunized with R Strains. (Three Rabbits Immunized with S<sub>111</sub> Are Also Included)*

Number of rabbits	Immunized with	Infected with Pneumococcus Type III	Route of infection	Number died	Number survived
1	R <sub>1</sub>	0 01	Intravenous	0	1
3	S <sub>111</sub>	0 1 0 01	" "	1* 0	0 2
8	R <sub>2</sub>	0 1 0 01	" "	1† 0	5 2
4	R <sub>2</sub>	0 1 0 01	Intraperitoneal "	1‡ 0	0 3
6	R <sub>2</sub>	0 1 0 01	Intradermal "	0 0	2 4
Total 22				3	19
9	Normal controls	0 000001 0 000001 0 000001	Intravenous Intraperitoneal Intradermal	6 1 2	0 0 0
Total				9	0

\* Animal died 8 days after infection.

† Animal died 5 days after infection.

‡ Animal died 7 days after infection. Controls receiving test dose of culture died within 36 hours.

### *Active Immunity.*

Rabbits were tested for resistance to infection by the injection of organisms intravenously, intraperitoneally, and intradermally (Table I). Blood cultures were taken at frequent intervals in order to observe the duration and degree of the bacteremia.

*Rabbits Infected Intravenously.*—Of 12 rabbits infected intravenously, 5 of which received 0.01 cc., and 7 0.1 cc. each of virulent Type I culture, 10 survived. The 2 which finally succumbed, lived 5 and 8 days respectively, whereas normal rabbits receiving the same dose died in 24 to 48 hours. As evidence of the duration of the immunity, 2 of the rabbits in this group were tested 4½ months after the last immunizing dose and found to be resistant. As demonstrated by blood cultures, organisms persisted in the circulation for 3 to 6 days, increasing and decreasing in number irregularly until their final disappearance. Even in the two fatal instances, the animals possessed some degree of partial immunity as revealed by the fact that both lived several days longer than the controls, that neither suffered an overwhelming septicemia, and that at autopsy each showed evidence of attempted localization in the form of pleurisy and pericarditis. A bacteremia characterized by an irregular course was previously shown to occur when R immunized rabbits were infected with a rabbit virulent strain of Type III pneumococcus (1). This form of bacteremia appears to be characteristic of the benign blood infection occurring when rabbits, immunized with R cells, are infected intravenously with any of the specific types of pneumococcus and suggests a similarity in the mechanism of recovery in each instance.

*Rabbits Infected Intraperitoneally.*—Four rabbits, immunized with R<sub>2</sub> organisms, were infected intraperitoneally; 1 received 0.1 cc. and 3 received 0.01 cc. of Type I pneumococci. The latter 3 animals survived; the one injected with 0.1 cc. lived 7 days. In the 3 animals which survived, only a few bacteria were transiently present in the blood stream. The duration of cocci in the peritoneal cavity was not determined.

*Rabbits Infected Intradermally.*—Type I organisms in doses of 0.01 cc. and 0.1 cc. were introduced intradermally into 6 rabbits which had previously been immunized with R<sub>2</sub> cells. All 6 animals survived. The local lesion developed rapidly; in 24 to 48 hours it appeared fulminating, usually reddish purple, edematous, spreading ventrally in a well defined and elevated band and forming a boggy pouch of edema over the more dependent portions of the abdominal wall. Areas of ecchymosis were commonly present. Normal rabbits reacted with a similar lesion, although succumbing to the infection. Furthermore, blood cultures revealed a striking difference in the course of the infection in normal and immunized animals. The blood stream of immune rabbits either remained sterile or contained only a few organisms transiently present. In sharp contrast, normal rabbits developed, within a few hours, a blood infection which increased rapidly in severity until death ensued from an overwhelming septicemia. Recently Goodner (5) has reported results obtained following intradermal injection of Type I pneumococci. The lesion which he describes as occurring in normal rabbits is identical with the inflammatory reaction encountered in the animals used in these experiments. Results which have been obtained following the intradermal inoculation of pneumococci into normal, type-specifically immune, and R immunized rabbits will be reported later in a separate communication.

In addition to the rabbits immunized with R organisms and tested for resistance to infection with Type I, 3 rabbits which had received similar preliminary injections were infected intravenously with virulent Type II pneumococci. They survived 10,000 lethal doses. The character of the blood infection and the process of recovery were similar in all respects to those already observed in the case of infection with Types I and III.

From the results of these experiments with Type I and Type II pneumococci, and from those reported (1) using Type III, it may be concluded that adequate immunization of rabbits with R pneumococci stimulates the development of active immunity which is effective against any of the fixed types. A consideration of these results and their possible significance will be presented in the discussion.

### *Passive Immunity.*

In the course of an analysis of the immunity induced by repeated injections of R pneumococci, experiments have been carried out to determine whether this form of resistance is passively transferable. Whole citrated blood and serum of rabbits which have acquired resistance through immunization with R cells have been passively transferred to normal rabbits and also to mice. The R strain used for immunization was derived from Type II S culture. The infecting organisms were virulent S strains of Type I or Type III. These precautions were taken in order to minimize the possible participation of type-specific antibodies. The blood for transfusion was drawn 9 to 14 days after the last immunizing dose.

In the first experiments the procedure was to transfer whole blood or its constituents from resistant to normal rabbits and 24 hours later to inject the recipients with virulent pneumococci. An example of the results obtained following the passive transference of whole citrated blood, plasma, cells, and serum is given in Table II.

A description of this experiment will serve as an illustration of the method employed and the results obtained.

From the ear vein of an R immunized rabbit 20 cc. of blood was allowed to drop into a tube containing 0.5 cc. of a saturated solution of sodium citrate. This made in final dilution approximately a 2 per cent sodium citrate solution. Immediately upon obtaining the desired amount, the blood was injected by means of a syringe into the ear vein of a normal rabbit. In making transfusions from rabbit to

rabbit no precaution was taken with regard to blood grouping. Sometimes immediately after the operation the recipient would show evidence of shock, characterized by clonic and tonic muscular spasms. Complete recovery usually occurred in 3 to 4 minutes. In one instance death ensued and in 2 other animals permanent paralysis of the hind limbs resulted. 20 cc. of blood similarly collected in citrate were separated by centrifugation into plasma and cells. An equal amount without citrate was allowed to clot and the serum collected. Sterile

TABLE II.

*Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood, Plasma, Cells, and Serum of Rabbits Immunized with R Pneumococci.*

*1. Protocol of Course of Bacteremia in Anti-R Donor and Recipients.*

Time of blood culture	Number of colonies per unit of blood								Control 0.0001 cc. Type III
	Anti-R donor	Anti-R recipients				Normal donor	Normal recipients		
		Whole blood	Plasma	Serum	Cells		Whole blood	Serum	
2 hrs.	1	42	86	93	113	∞	20	1000	D
6 hrs.	13	5	0	40	13	∞	2	816	
10 hrs.	1	9	15	62	142	∞	23	∞	
20 hrs.	58	15	7	12	518	D	148	∞	
24 hrs.	142	1	2	38	∞		272	D	
30 hrs.	32	0	29	116	∞		522		
48 hrs.	1	1	1	25	∞		∞		
72 hrs.	0	1	1	3	∞		D		
96 hrs.	4	0	12	14	D				
5 days	0	0	1	8					
6 days	0	0	8	1					
9 days	0	0	0	0					
11 days	0*	0*	0*	0*					

0.1 cc. of rabbit virulent strain of Type III used as infecting dose.

D indicates death of the animal.

S indicates survival of the animal.

Numerals represent number of colonies per unit of blood.

precautions were observed throughout the procedure. 20 cc. of whole blood, or its equivalent in plasma, cells, or serum were then injected intravenously into normal rabbits. 24 hours later these rabbits were infected intravenously with 0.1 cc. of a rabbit virulent strain of Type III. Normal rabbits which had received comparable amounts of whole blood or serum from other normals were similarly infected. Other normal rabbits without preliminary treatment were infected with the maximal test dose and the minimal lethal amount of culture.

From Table II it may be seen that whole blood, plasma, and serum from resistant rabbits afforded protection against 1000 lethal doses of Type III pneumococcus, whereas blood cells alone were inadequate. Controls receiving normal blood or serum were unprotected. Tabulation of the number of organisms in the blood cultures reveals the fact that the resistant rabbits continued to have pneumococci in varying numbers in the blood stream from 3 to 6 days before permanent sterility was attained. Rabbits receiving normal blood, on the other hand, although possessing a slight initial capacity to reduce the number of circulating bacteria were unable to cope with the subsequent rapid increase, and died of an overwhelming septicemia.

Repetitions of protection experiments of this character using virulent Type I pneumococci instead of Type III gave results equally definite, indicating that, as in the case of active immunity, passive protection of rabbits is not limited as to type of infecting pneumococcus.

From these experiments it is established that resistance induced in rabbits by immunization with R pneumococci can be passively transferred to normal rabbits. In titering the amount of blood necessary to confer passive protection it was found that 15 to 20 cc. were necessary against doses of culture as high as those constantly employed, *i.e.* 1000 lethal doses of Type III or 10,000 to 100,000 of Type I. This quantity of blood was regularly used in all subsequent experiments. In Table III the total number of transfusions and the results are recorded. It may be seen that, with the amount of blood transfused and the dosage of culture kept constant, the time elapsing between transfusion and injection of organisms has been varied from 1 hour to 21 days. Of 5 rabbits infected within 5 hours of the time of transfusion 4 died and 1 survived. Twenty-three animals were infected 1 to 7 days after transfusion and of these 18 recovered. One rabbit infected 14 days after transfusion, survived, and of 5 in which an interval of 3 weeks elapsed, 3 recovered.

With the use of 8 to 10 cc. of serum, an amount comparable to that contained in 15 to 20 cc. of whole blood, protection was demonstrable but the results were somewhat less striking. In Table III the results of protection tests by the use of serum are presented. Of 5 rabbits infected 1 to 5 hours after serum administration, 4 survived. With an interval of 1 to 7 days, out of 9 animals tested, 4 survived; in 3 in-

TABLE III.

*Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood and Serum of Rabbits Immunized with R Pneumococci.*

*2. Summary of Results of Passive Protection Tests in Rabbits.*

Rabbit No	Amount of blood	Time interval	Infection with	Result
	"			
1	20	1 hr.	0 1 cc. Type I	D 6 days
2	20	2 hrs.	0 1 " " "	S
3	16	4 "	0 5 " " III	D 4 days
4	15	4 "	0 5 " " "	" 4 "
5	15	5 "	0 5 " " "	" 4 "
6	20	24 "	0 1 " " I	" 4 "
7	15	24 "	0 1 " " "	S
8	20	24 "	0 5 " " III	"
9	20	24 "	0 2 " " "	"
10	20	24 "	0 2 " " "	"
11	20	3 days	0 1 " " I	"
12	20	3 "	0 1 " " "	"
13	20	3 "	0 1 " " "	D 4 days
14	20	3 "	0 01 " " "	S
15	20	3 "	0 01 " " "	"
16	20	3 "	0 5 " " III	"
17	15	4 "	0 1 " " I	D 4 days
18	15	4 "	0 1 " " "	S
19	15	4 "	0 01 " " "	"
20	20	7 "	0 01 " " "	"
21	20	7 "	0 01 " " "	"
22	20	7 "	0 1 " " "	"
23	20	7 "	0 1 " " "	D 4 days
24	17	7 "	0 1 " " "	S
25	15	7 "	0 4 " " III	"
26	15	7 "	0 5 " " "	"
27	15	7 "	0 5 " " "	D 8 days
28	20	7 "	0 1 " " "	S
29	20	14 "	0 01 " " I	"
30	20	21 "	0 01 " " "	"
31	20	21 "	0 01 " " "	"
32	20	21 "	0 01 " " "	"
33	15	21 "	0 01 " " "	"
34	20	21 "	0 01 " " "	"

D indicates death of the animal.

S indicates survival of the animal.

TABLE III—*Concluded.*

Rabbit No.	Amount of serum	Time interval	Infection with	Result
	cc.			
35	10	1 hr.	0 1 cc. Type I	S
36	10	1 "	0 1 " " "	"
37	10	2 hrs.	0 1 " " "	"
38	10	4 "	0 5 " " III	D 7 days
39	10	4 "	0 5 " " "	S
40	10	24 "	0 1 " " I	D 10 days
41	10	24 "	0 5 " " III	" 3 "
42	8	24 "	0 5 " " "	" 7 "
43	10	24 "	0 1 " " I	S
44	12	3 days	0 1 " " III	"
45	8	7 "	0 4 " " "	"
46	8	7 "	0 5 " " "	D 7 days
47	10	7 "	0 01 " " I	" 2 "
48	10	7 "	0 01 " " "	S
49	10	14 "	0 1 " " "	D 4 days
50	10	21 "	0 1 " " "	" 4 "
51	10	21 "	0 1 " " "	" 2 "

stances where the time interval was longer than 7 days, none of the animals recovered.

In all of the experiments controls were used consisting of rabbits which received quantities of normal whole blood or serum equal to the amount of immune blood or serum transferred. Altogether 20 control rabbits were transfused with normal whole blood and 10 received injections of normal serum. They all died of pneumococcus septi-cemia.

Differences in the effectiveness of passive protection depending on the time elapsing between administration of serum or blood and the injection of the infecting organism may be noted in Table III. In those instances where serum was employed, protection appeared to be most effective, if only a few hours elapsed before the injection of organisms. On the other hand, when whole blood was transfused, protection was less striking when the interval was short than when the animals were permitted to rest 24 hours or longer before infection. The duration of the protection conferred by whole blood is evidenced by animals which survived pneumococcus infection 3 weeks after transfusion.

No definite conclusions can be drawn at the present time from the results concerning the time intervals employed in passive protection tests. The significant fact is that the circulating blood of rabbits immunized with R pneumococci possesses active principles which, when transferred to normal rabbits, confer upon the recipients protection against infection with virulent pneumococci.

TABLE IV.

*Comparison of Passive Protection of Mice and Rabbits by Serum of Rabbits Immunized with R<sub>2</sub> Pneumococci.*

Anti-R rabbit serum		Amount of culture Type I	Results	
			Rabbits	Mice
cc.		cc.		
10	*Interval of 2 hrs.	0.1	D 24 hrs.	
10		0.01	S	
10		0.01	S	
10		0.01	S	
0.5		0.00001		D 24 hrs.
0.5		0.00001		D 24 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 40 hrs.
		Controls		
None		0.000001	D 60 hrs.	D 24 hrs.
None		0.000001		D 24 hrs.
None		0.000001		D 30 hrs.

D indicates death and figures the number of hours before death of animal.

\* 2 hours following intraperitoneal injection of serum all animals were infected intraperitoneally with virulent culture in amounts indicated.

In contrast to the effective protection of rabbits just described, attempts to protect mice by the use of the same sera have been entirely negative. From Table IV it may be seen that serum which protected rabbits against 0.01 cc. did not protect mice against even 0.000001 cc. of culture. This was true in spite of the fact that the mice received a much larger amount of serum per unit of body weight than did the rabbits. Repeated attempts to protect mice with resistant rabbit's serum have failed regardless of whether the infecting organisms were



introduced simultaneously with serum or after intervals of 2, 6, 12, or 24 hours. The whole citrated blood of rabbits has been similarly tested and found to be without effect in mice.

It may be concluded, then, that the serum or whole blood of rabbits immunized by repeated injections of R pneumococci, although able to afford protection to normal animals of the same species, is incapable, under comparable conditions, of conferring protection upon animals of a foreign species—*i.e.* mice. These results are in striking contrast to those obtained with antipneumococcus sera which possess a high content of type-specific antibodies.

#### DISCUSSION.

The experiments recorded in the present communication demonstrate that a considerable degree of active immunity against Type I and Type II pneumococci may be stimulated in rabbits by repeated injections of R pneumococci. This form of resistance, elicited by R organisms which are devoid of type specificity, is effective in the absence of demonstrable *type-specific* agglutinins, precipitins, and antibodies passively protective for mice. In a preceding paper (1) it was reported that rabbits similarly treated are resistant to virulent Type III organisms; the present results with Types I and II establish the fact that immunity induced in rabbits by R strains is sufficiently broad to be effective against infection with each of the three specific types of pneumococcus. The Type III infections as previously pointed out, were characterized by a bacteremia which ran a prolonged course during which the number of circulating bacteria varied from time to time but eventually disappeared. The Types I and II infections encountered in the present experiments behave similarly. These facts are suggestive that in this form of immunity the mode of recovery from infection involves the same mechanism, or different mechanisms acting in a similar manner, against each type of pneumococcus. Furthermore, these results strongly imply that resistance under these conditions is dependent either upon other factors than those concerned in type-specific immunity, or upon the same factors operative in a different manner.

Although the majority of previous investigators have found that active immunity against pneumococcus infection is type-specific, the ex-

perimental conditions, either as to the species of animal or the method of immunization employed, have differed from those reported in this paper. Cecil and Blake (6) observed that in monkeys vaccination with living cultures of Type I conferred a certain amount of cross-immunity, the degree of effectiveness being subject to variation. The immunizing dose in their experiments consisted of *one* subcutaneous injection of either 0.001 cc. of virulent or 1 to 2 cc. of avirulent organisms. Wright (7) found that *one* preliminary intravenous injection of *S* pneumococci produced active immunity in rabbits effective only against homologous organisms. Barach (8) employing mice gave *one* intraperitoneal injection of *S* organisms and obtained strict type-specific immunity.

In the experiments here reported the active resistance which was stimulated by immunization with *R* pneumococci and which was found to be effective against all the fixed types, was obtained by a more prolonged series of injections. The process of immunization comprised 18 intravenous injections carried out over a period of 6 weeks according to the method described by Cole and Moore (2). Although the degree of immunization necessary to incite non-type-specific immunity has not been determined, it has been found that one injection of from 5 cc. to 25 cc. of *R* culture is insufficient.

The purpose of these experiments has been an attempt to understand the factors underlying the resistance. Since this form of cross-immunity can be induced by pneumococci devoid of type-specific properties, it seems highly probable that a mechanism of a different order from that involved in type-specific resistance is implicated. Work is in progress at the present to define more clearly the points of similarity and difference between these two forms of acquired resistance.

Having determined the presence of active immunity in rabbits previously treated with *R* cells, consideration has been given to passive immunity. Since one of the chief characteristics of type-specific immunity is the passive protection afforded animals of any species by an immune serum of the homologous type a study of the possibility of passively transferring this non-specific resistance has been carried out. It was found that whole blood or serum of *R* immunized rabbits protected normal rabbits against infection with virulent Type I and

Type III pneumococci.\* As a rule, blood in 15 to 20 cc. amounts was found to afford a more solid resistance against a given infecting dose than the equivalent amount of serum.

Under the experimental conditions described the most striking results have been obtained when an interval of time elapsed between the transfusion and the injection of organisms. The exact significance of these relations has not as yet been sufficiently studied to justify final conclusions. However, it can be stated at the present time, that the immunity elicited by repeated injections of R pneumococci in rabbits can be passively transferred by the circulating blood to normal rabbits. This is evidence that there is present in the circulation of resistant rabbits either protective substances in an active state or something which stimulates the mechanism of resistance in the transfused animal.

Attempts to confer passive protection on mice under similar conditions have been uniformly negative. This failure is in sharp contrast to the positive results always obtained in mice with type-specific sera, and is further evidence of a difference in the mechanism involved in each instance.

#### SUMMARY.

1. Rabbits, vaccinated by repeated intravenous injections of suspensions of heat-killed R pneumococci, acquire a marked degree of active immunity to infection with the virulent S forms of *Pneumococcus* Types I and II. Previously (1) it was shown that the immunization of rabbits with R cells induces active resistance to Type III infection. This immunity is effective when the infecting organisms are injected either intravenously, intraperitoneally, or intradermally.

2. Whole citrated blood or serum of rabbits immunized with R pneumococci, under the experimental conditions described, is capable of passively protecting normal rabbits against Type I and Type III infection. Whole blood appears to be more effective than an equivalent amount of serum.

3. Passive protection of mice by the use of whole blood or serum of

\* Resistance to Type II was not tested by reason of the fact that an R strain originally derived from Type II S culture was used for immunization. It seemed desirable to minimize the possible participation of type-specific substances.

the immune rabbits has been entirely ineffectual. This is in striking contrast to the results obtained with type-specific immune serum.

4. This form of acquired resistance to pneumococcus infection, elicited by R organisms which are devoid of type specificity, and exemplified in animals whose sera possess no demonstrable type-specific antibodies, has many characteristics strongly suggesting that the underlying mechanism differs from that concerned in type-specific immunity.

#### CONCLUSION.

A broad immunity against infection with virulent S pneumococci (Types I, II, and III) can be induced in rabbits by vaccination with the degraded R strains of pneumococcus. This form of active resistance is effective in the absence of demonstrable type-specific antibodies, and may be passively transferred to normal rabbits by the blood of the immunized animal.

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## RHEUMATIC FEVER AS A MANIFESTATION OF HYPER- SENSITIVENESS (ALLERGY OR HYPERERGY) TO STREPTOCOCCI.

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The object of this communication is to present briefly the evidence for a theory that the symptom-complex included under the term rheumatic fever is a manifestation of hypersensitiveness to streptococci. In many quarters the dictum that this disease is due to streptococci has been accepted as proven; but up to the present time, so far as we are aware, no one has advanced a satisfactory explanation of the differences between the manifestations of rheumatic fever, which is suspected of having these organisms as causative agents, and those of subacute bacterial endocarditis, which is known definitely to be due to *Streptococcus viridans* in most instances. We feel that the correlation of some experimental work upon animals which we have been pursuing for several years with certain phenomena seen in the clinic offers a satisfactory explanation of the above-mentioned differences.

Several years ago we<sup>1</sup> presented a comparison of the similarity of many of the manifestations of rheumatic fever with those of tuberculosis and syphilis; almost simultaneously similar views were presented in France by Bezançon and Weil.<sup>2</sup> If we include under the term rheumatic fever the form of heart disease that is now known often to be due to a subacute or chronic rheumatic infection, the similarity is made more evident. In all three diseases there is usually an initial lesion, which when present is followed shortly by distinct enlargement of the satellite lymph nodes. In all, the serious manifestations occur at a distance from this lesion and usually are multiple in distribution. In tuberculosis, although the majority of the population is infected, only relatively few persons ever show clinical symptoms. In both rheumatic fever and tuberculosis a rise in the standard

of general living conditions has been accompanied by a decreasing incidence of the diseases. A number of workers<sup>3</sup> have called attention to a similarity in the familial incidences of these two infections. In all three diseases there is a decided tendency to relapses which seems to be due to a break-down of a partial immunity. In all the histopathological pictures of the typical lesion is that of a granuloma, the histogenesis of which in tuberculosis and syphilis has been definitely proven to be allergic in nature. This allergic lesion can be considered an effort on the part of the body to restrict the activity of the micro-organism to these local areas. The tubercle has the same general characteristics throughout the life of the individual, although there may be special features of the tuberculous infection in certain tissues or organs. While the different syphilides may seem to have decidedly different characteristics these variations, may be considered quantitative rather than qualitative. In both tuberculosis and rheumatic fever the presence of lesions in the walls of the serous cavities or joints is accompanied by the outpouring of serofibrinous exudate. In both rheumatic fever and syphilis there is a marked tendency to focal endo- and perivascular involvement; and both infections are prone to attack the heart. It is true that differences can be found in the three diseases; but it is in these differences that there reside the features which bring about the peculiar pictures of the respective maladies. On the other hand, in individual cases it is sometimes difficult to decide whether one or another type of infection is active until specific differential diagnostic measures can be applied.

For several years we attempted to induce in lower animals lesions bearing some resemblance to those of rheumatic fever by inoculating them directly with material from patients likely to harbor the virus, and also with cultures made in a variety of ways from these materials; but no success attended these efforts.<sup>4,5,6</sup> The only microorganisms appearing with any regularity were different types of streptococci.<sup>7</sup> Thus the observations of others<sup>8</sup> were confirmed; but the streptococci were recovered from the blood cultures or lesions in only a relatively small proportion of our patients; and, furthermore, when recovered they did not conform to any one cultural or immunological type, but were, on the contrary, distinguished by their heterogeneity.<sup>7</sup>

It <sup>9,10</sup> was then observed that intracutaneous inoculation of rabbits

with suitable doses of certain strains of *Streptococcus viridans* induced a change in the reactivity of the tissues of the animals which has been subsequently shown to resemble very closely tuberculin allergy.<sup>11</sup> Recently we<sup>12</sup> have also demonstrated that a similar hypersensitiveness can be induced with certain strains of indifferent streptococci similar to those claimed by Small<sup>13</sup> and by Birkhaug<sup>14</sup> to be the specific causative agents in the disease; some preliminary observations have indicated, furthermore, that intracutaneous inoculation with very small doses of certain hemolytic streptococci markedly increases the reactivity of the animals to subsequent inoculation with green-producing or indifferent strains. We have employed three different tests for the presence of the hypersensitive state: (1) cutaneous, (2) ophthalmic, and (3) lethal. Upon reinoculation of the animal with the same sized doses the degree of reactivity is found to be heightened up to a certain limit, so that lesions three or four times the size of the original one result. Doses, such as 0.0001 to 0.000001 cc. that fail to induce any grossly visible lesions in normal animals, induce lesions from 10 to 15 mm. in diameter in hypersensitive ones, and as the degree of hypersensitiveness increases the lesions increase in height and often are characterized by considerable edema. In color they resemble somewhat a lupus nodule or a nodular syphilide. If the anesthetized cornea of a hypersensitive rabbit is lightly scarified and a drop of sedimented culture is instilled into the conjunctival sac there appear within the following few days conjunctivitis and keratitis, the intensity of which is conditioned by the degree of hypersensitiveness of the animal and by certain properties of the culture. Varying with its intensity this eye reaction persists from five to fifteen days. In our experience normal rabbits have never shown keratitis when inoculated in this manner with nonhemolytic streptococci, but have shown similar ophthalmic reactions following inoculation with living hemolytic streptococci. When hypersensitive animals are inoculated intravenously with doses of broth culture that are well tolerated by normal rabbits they sicken and in the course of from twenty-four to forty-eight hours many of them die, and show at autopsy enlarged and hemorrhagic lymph nodes and thymus, and hemorrhages in the bone-marrow. Many show in addition hemorrhagic lesions of the

heart and lungs. This closely resembles the well known condition of tuberculin shock.

The lack of strain specificity is an interesting feature of this state. While hypersensitiveness is usually best made evident by testing with the same strain that was used in sensitizing the animal, other more or less distantly related strains can be used for testing. The more highly sensitive the animal the broader is the zone of reactivity, so that a rabbit highly sensitized with a *Streptococcus viridans* shows a positive ophthalmic reaction to inoculation with indifferent streptococci, and large skin lesions following small doses of such cocci.

While the hypersensitive state can be most readily induced by intracutaneous inoculation, it also follows inoculation into other tissues and even into the paranasal sinuses. If, on the other hand, inoculation is by the intravenous route this type of hypersensitiveness does not ensue, and the animal shows no ophthalmic reaction following corneal inoculation and no lethal reaction following intravenous inoculation.<sup>15</sup> In this respect it might be considered to react like a normal animal; but inoculation of streptococci into its skin shows that it is not normal but immune. By titration of the inoculum it is found that for the production of a lesion of a given size it is necessary to inject the immune animal with a dose from ten to one hundred times greater than that which will produce a lesion of the same extent in the normal animal. Furthermore, a given medium-sized inoculum, which in the normal animal produces a soft or somewhat infiltrated lesion of moderate size, results in the immune animal in the production of a small and distinctly hard nodule, while in the hypersensitive animal a very large and often markedly edematous lesion appears. Thus three states are demonstrable: the normal, in which a definite lesion is produced which recedes after a few days but which again increases in size at some time between the seventh and fourteenth days; the hypersensitive, which is characterized by over-reaction to reinoculation; and the immune, in which a nodular lesion without preliminary edema appears at the site of local inoculation.

Histologically distinct differences are also distinguishable in the three types of response. With a properly selected inoculum the normal animal shows moderate tissue destruction followed by moderate edema, out-pouring of a fair number of leukocytes, and later the



usual increase in macrophages and histiocytic reactions; the hypersensitive animal shows more tissue destruction, more intense edema and out-pouring of leukocytes, followed in a few days by a more intense histiocytic response than is seen at a corresponding period in the normal animal; the immune animal shows a minimum of tissue destruction, little if any edema, and only a few polymorphonuclear leukocytes, but, on the other hand, a very rapidly appearing histiocytic reaction. Thus, while both the hypersensitive and immune reactions can be considered as expressions of an effort on the part of the body to limit the activity of the invading microorganisms to a local area, there is present in the first instance, a maximum of tissue destruction, and in the second a minimum. As would be expected, the scarring following the former is much greater than that following the latter.

The intensity of the two states can be increased within limits by repeated inoculation; but, of course in each instance the inoculation must be made in the proper manner. The hypersensitive state can also often be maintained for fairly long periods without repeated inoculations if a suitably infected agar focus is introduced subcutaneously. If subcutaneous inoculation is not continued or if the focus is removed the hypersensitiveness gradually diminishes. In some instances where the degree of hypersensitiveness was not initially very great the animal reacted in a normal manner to reinoculation after a period of eight or ten weeks; in more highly sensitized animals the rate of return toward normal was slower. It has also been possible to reduce the hypersensitiveness by gradual and mild intravenous immunization.

Our thesis is that the manifestations of rheumatic fever are those of a state of hypersensitiveness on the part of the body to streptococci or some product of streptococci, and that the absence of severe focal reactions of an exudative nature in subacute bacterial endocarditis is due to an immune condition of the tissues in this disease.<sup>16</sup> Most patients with rheumatic fever present evidence of a persisting focus of infection somewhere in the body; usually in the form of chronic tonsillitis, sinusitis, or other infection of the upper respiratory tract. These foci, in our opinion, represent areas where the sensitizing substance is produced and whence it is distributed to the entire body, as well as localities where bacteria may be fed into the blood stream.

Lewis and Grant<sup>17</sup> contended several years ago that nonhemolytic streptococci were probably frequent invaders of the blood stream, but that only under special circumstances did they give rise to endocardial lesions. The recent observations of Kugel and Epstein<sup>18</sup> substantiate the opinion that these microorganisms are often transient inhabitants of the blood stream; but this does not mean that in all such instances the streptococci are necessarily pathogenic. Indeed, in our investigations we have encountered many strains of non-hemolytic streptococci with which we have been unable to induce hypersensitiveness and which have been practically incapable of eliciting signs of allergy in hypersensitive rabbits. Furthermore, strains that have been repeatedly transferred on artificial media have lost their sensitizing capacity.

Birkhaug,<sup>14</sup> Kaiser,<sup>19</sup> and Strauss<sup>20</sup> have recently presented evidence indicating that patients with rheumatic fever show marked skin reactions following the introduction of certain products of streptococci into their cutaneous tissue. The first two observers employed filtrates of indifferent streptococci; Strauss used Pondorf's cutivaccine. Mackenzie and Hangar<sup>21</sup> have found that many persons show cutaneous reactions to filtrates or extracts of both hemolytic and green streptococci, but that this reactivity appears to be a function of age rather than a characteristic of any one disease. It is interesting to note from their published cases that many of the strong reactors were suffering from rheumatic fever. We have found that most of our patients with active rheumatic fever react strongly to filtrates of both indifferent and green-producing streptococci and to nucleoproteins from all types of streptococci. The patients with subcutaneous nodules or long-standing heart disease have shown the strongest reactions. We also have found that patients with other diseases sometimes give similar reactions; hence this type of skin reaction cannot be considered diagnostic of rheumatic fever. The important point is that so great a proportion of patients with this disease react.

We<sup>22</sup> have also tested a series of patients with intravenous injections of very small doses of streptococcus vaccines or nucleoproteins and have found that they undergo delayed febrile reactions which correspond closely to the late reactions seen in tuberculous patients or animals following a tuberculin test. Here again the severity of the

reaction was roughly proportional to the chronicity and severity of the active disease. The lack of specificity of these reactions may be explained to a certain degree by the recent indications that possibly the disease rheumatic fever may not be so closely linked to polyarthritis as was formerly thought. From this angle the recent statistics of VonGlahn<sup>23</sup> showing the large proportion of patients dying with active rheumatic heart disease but without history of arthritis are most illuminating. Many patients with Sydenham's chorea do not have evidence of rheumatic carditis or polyarthritis, yet the viewpoint that all are rheumatic is steadily gaining ground.

The theory of Escherich and Schick<sup>24</sup> that the late complications of scarlet fever are allergic in nature may be adduced in support of our viewpoint. Among these late complications are polyarthritis and verrucous endocarditis closely resembling those of rheumatic fever. It is well known and has been recently emphasized by Hector<sup>25</sup> that an attack of scarlet fever in a patient who has previously suffered from rheumatic fever is very liable to induce a new attack of carditis. In our opinion, this is due to a superimposition of a streptococcus allergy on a preexisting allergy with the result that the hypersensitive state is raised to a point where pathological and clinical evidences appear. Indeed, Schmorl<sup>26</sup> has described submiliary nodules similar to Aschoff bodies in the myocardium of a patient dying from scarlet fever. Fahr<sup>27</sup> as a result of a more extensive study, states that the perivascular reactions found in the hearts of fatal cases of scarlet fever differ from typical Aschoff bodies only in not containing giant cells, and that myocardial lesions are often found in rheumatic hearts which cannot be distinguished from those of scarlatinal myocarditis. It is possible that the difference in the chronicity of the two diseases may explain the pathological differences. Indeed, in the hearts of patients dying very early in an attack of rheumatic fever we have found relatively fewer giant cells and more marked exudation than are observed in more long-standing myocarditis. In their very complete studies of the cardiac and vascular reactions in rheumatic fever Pappenheimer and VonGlahn<sup>28,29</sup> have found numerous examples where polymorphonuclear cells and smaller types of mononuclears were as frequently present as were giant cells.

The recent studies of Siegmund,<sup>30</sup> Oeller,<sup>31</sup> Kuczynski and Wolff,<sup>32</sup>

and Dietrich,<sup>33</sup> show how important are the endovascular reactions in various types of infection for an understanding of the course of many diseases. In most of these studies no clear distinction was made between a hypersensitive state and an immune state of the tissues such as we have drawn. In Dietrich's experiments alone were the animals sensitized exclusively by introducing the bacteria into the tissues. Of course in tuberculous infections focal lesions always result from any mode of infection; hence the reactions of tuberculous animals cannot be compared strictly with those of animals in which the immunization has been induced by intravenous inoculations of other bacteria. We feel that the very intense vascular reactions described in rheumatic fever by Pappenheimer and VonGlahn<sup>34</sup> and more recently by Kugel and Epstein,<sup>35</sup> compared with the relatively mild reactions observed by Siegmund and others in their experimental animals, are explainable on the basis of differences in reactivity in the tissues of hypersensitive and immune animals. These are, however, questions for additional study to decide.

The wide range of reactivity of the hypersensitive state can be used to account for the pathogenic rôle of the different streptococci which have been recovered from blood or lesions of rheumatic fever patients. We have occasionally isolated two distinct strains from one patient at different times and in one instance two types from one group of subcutaneous nodules removed at biopsy. As already noted, a survey of the literature of the last two or three decades indicates that many different strains of streptococci have been recovered from patients with this disease, and this heterogeneity has been a perplexing element in attributing any etiological rôle whatever to streptococci.

Recent studies of Dochez and Stevens<sup>36</sup> indicate that there are two types of hypersensitiveness to streptococci; one to a neutralizable "toxin" and the other, probably to some other bacterial product, which is not neutralizable. Zinsser and Grinnell,<sup>37</sup> and Mackie and McLachlan<sup>38</sup> obtained sensitization of the second type by injecting guinea-pigs in a suitable manner. The animals of the latter observers were sensitive to a number of heterologous streptococci. Birkhaug<sup>34</sup> claimed that he could produce a type of hypersensitiveness in animals with filtrates of indifferent streptococci, and that it was possible to neutralize the "toxic filtrate" with immune serum. In our

experience<sup>39</sup> it has been impossible to obtain comparable neutralization in patients in the acute stages of rheumatic fever; rather, the mixture of immune serum and filtrate has induced more marked reactions than did the filtrate alone. The hypersensitive state of these patients seems to be more comparable to that observed after sensitization with whole bacteria than after sensitization with a neutralizable "toxic filtrate." Zinsser,<sup>40</sup> who has recently advanced a hypothesis that rheumatic fever may be an allergic disease, thinks that the antigen which induces the allergic state and which irritates the tissues to produce arthritis is an autolytic product of streptococci, and cites some experiments performed by him and Grinnell<sup>41</sup> with autolysates of pneumococci to support his opinion. Menzer<sup>42</sup> in 1902, in an endeavor to explain the peculiar action of his serum in rheumatic fever, advanced a theory which in many respects resembles the modern concept of allergy. Weintraud<sup>43</sup> also advanced a hypothesis that rheumatic fever corresponded with the type of hypersensitiveness seen in serum disease. *While these two diseases have some features in common, they have points of dissimilarity which are explainable on the basis of the differences between protein hypersensitiveness and bacterial allergy.*

In one other respect an analogy between the condition of allergic rabbits and that of rheumatic fever patients has been demonstrable. Just as it has been possible to reduce or extinguish the allergic state of the rabbits by intravenous injection of streptococci or streptococcal nucleoproteins, it has likewise been possible to cause a reduction in the intensity of the febrile and skin reactions of some patients by careful intravenous immunization. This work is still in its early stages, and is cited only in order to give as complete evidence as we have been able to obtain.

We are unable to answer definitely the question why all individuals who show hypersensitiveness to streptococci do not have rheumatic fever. A comparable condition exists in many persons who give a positive tuberculin reaction and still do not have signs of active infection. Only a small percentage of the patients suffering from scarlet fever develop nephritis. Some have fever alone in the third or fourth week of the disease as evidence of the existence of bacterial allergy; others have in addition angina, or angina and adenopathy.

It is probable that two factors at least must be active in order to elicit the symptoms of disease: viz., a high degree of hypersensitiveness plus a sufficient concentration of antigenic substance in the tissues to stimulate local reactions. It is not impossible that additional factors, included under the general term predisposition and as yet not clearly defined, play a rôle in determining just which individuals will develop the phenomena that we recognize as making up the disease.

CONCLUSION. The theory is advanced that the pathogenesis of rheumatic fever can be explained by the existence in certain individuals of a condition of hypersensitiveness (allergy or hyperergy) to streptococci resulting from repeated low-grade infections or from the persistence of foci of infection in the body. When under suitable circumstances streptococci or products of streptococci are disseminated to the tissues these tissues over-react and the characteristic picture of the disease results.

When, on the other hand, a state of immunity without this peculiar hypersensitiveness exists the dissemination of streptococci results in a minimum of injury to the tissues and the characteristic phenomena of the disease fail to appear.

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## SKIN REACTIONS OF PATIENTS WITH RHEUMATIC FEVER TO TOXIC FILTRATES OF STREPTOCOCCUS.

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Within recent years, the development of knowledge concerning the capacity for many strains of hemolytic streptococci to produce in broth cultures a substance which has certain resemblances to a soluble bacterial toxin has opened the field for study of a similar substance in other forms of streptococci. Following the discovery by Small<sup>1</sup> and Birkhaug<sup>2</sup> of indifferent streptococci in blood cultures of some patients with rheumatic fever, the latter observer investigated the capacity of these peculiar cocci to produce this kind of toxic filtrate, and he found that a much higher proportion of indifferent strains produced such toxic filtrates than did green streptococci. He also found that persons who had suffered from rheumatic fever were more susceptible to intracutaneous injections with these toxic filtrates than were other groups of persons, and consequently suggested that these micro-organisms might be the specific etiologic agents in this disease. Later, Kaiser<sup>3</sup> reported a study of the skin reaction of a much larger number of children who had had rheumatic fever and confirmed Birkhaug's original observations. In this study, however, a report was not made of the capacity of these children to react to boiled filtrate; and while a comparison of their reactions to the Schick and Dick tests is reported, study of a toxic filtrate of green streptococci is not recorded.

Because of finding green streptococci in the blood or lesions of patients with rheumatic fever by a number of observers, it seemed to

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2. Birkhaug, K. E.: *J. Infect. Dis.*, 40: 549, 1927.

3. Kaiser, A. D.: *J. Infect. Dis.*, 42: 25, 1928.

us that the study of skin reactivity to toxic filtrates of various streptococci warranted more extensive investigation; hence the present work was undertaken.

#### MATERIAL AND METHODS.

The clinical material consisted of children with rheumatic heart disease who had been under observation for several years in the wards and outpatient department of the New York Nursery and Child's Hospital, children with similar disease who were inmates of the Irvington Convalescent Home and patients with active rheumatic fever in the wards of the Hospital of the Rockefeller Institute. In this material were included practically all of the usual manifestations of rheumatic fever: active and latent cases and cases in which recovery probably occurred were represented. Data on control cases were obtained from other departments of these institutions.

As a standard toxic filtrate, we used a preparation furnished us by Dr. Birkhaug. It was a ten-day filtrate in Douglas tryptic broth of strain RF1B. We attempted to prepare similar filtrates from a number of strains of indifferent streptococci including RF1B, but were unable to obtain solutions that gave as uniformly satisfactory reactions even though heavy growths of the bacteria occurred in the broth. It was evident, however, that different strains varied greatly in their capacity to produce "toxins," for different strains incubated under identical conditions produced solutions of varying strength. It seemed, therefore, that because satisfactory filtrates were obtainable from only selected strains of indifferent streptococci, it might be possible to obtain good "toxin" producers among some strains of green streptococci. We had in the laboratory strains V110A, isolated from a subcutaneous rheumatic nodule, and P63, which was isolated in pure culture from an apical abscess of a tooth extracted from a patient with nodules; both of them produced fairly satisfactory toxic filtrates—in fact, filtrates as good as any we were able to produce from indifferent streptococci. During the course of this work, Dr. Birkhaug informed us that better filtrates resulted from cultures incubated for from ten to twelve days than from those incubated six days. We were able to confirm his statement, and this suggested that the differences in the strength of the filtrates of the various streptococci might be due in part to differences in time of death and ease of autolysis. Zinsser and Grinnell<sup>4</sup> asserted that the allergizing substance from many bacteria is a result of a peculiar type of autolysis. We soon found that indifferent streptococci died much sooner in tryptic broth cultures than did green streptococci, and that films made on

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4. Zinsser, H., and Grinnell, F. B.: *J. Bact.*, 14: 401, 1927.

successive days showed an increasing number of gram-negative and involution forms. In some previous work it was found that autolysis of green streptococci was difficult to obtain. Hitchcock,<sup>5</sup> moreover, determined that most strains of indifferent streptococci easily underwent dissolution in sodium hydrate. We were also able to observe autolysis of indifferent streptococci incubated in salt solution for two weeks; such autolysates, when filtered, however, did not give as

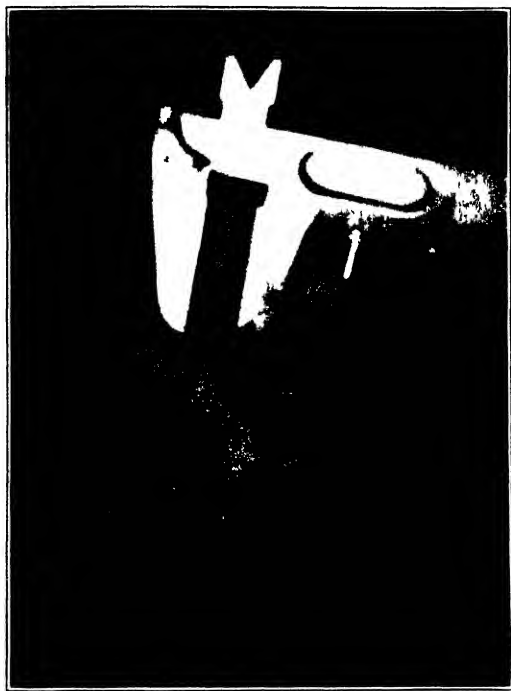


FIG. 1.—Type of outside diameter-caliper used to measure lesions.

good skin reactions as were obtained with simple broth filtrates and were therefore not used extensively.

The broth filtrates were diluted to the desired strength with normal salt containing 0.5 per cent phenol. In most of the work fresh dilutions were made each week, but in the latter part, dilutions two or three weeks old were employed because it was found that they did not deteriorate in this period. Using tuberculin

5. Hitchcock, C. H.: *J. Exper. Med.*, **48**: 393, 1928.

syringes and fine needles, we made injections of 0.1 cc. of the given dilutions into the skin of the mesial surface of the forearm or arm. The lesions were measured with a special outside adjusting caliper, as shown in figure 1, and the two longest diameters recorded in millimeters. In most instances the lesions were round or only slightly elliptic; hence, for purposes of comparison the sum of the two measured diameters was divided by 2, which gives the average diameter of a lesion. By recording the actual size of the lesion in this manner, all subjective impressions as to size, which are usually recorded by a different number of plus marks, were

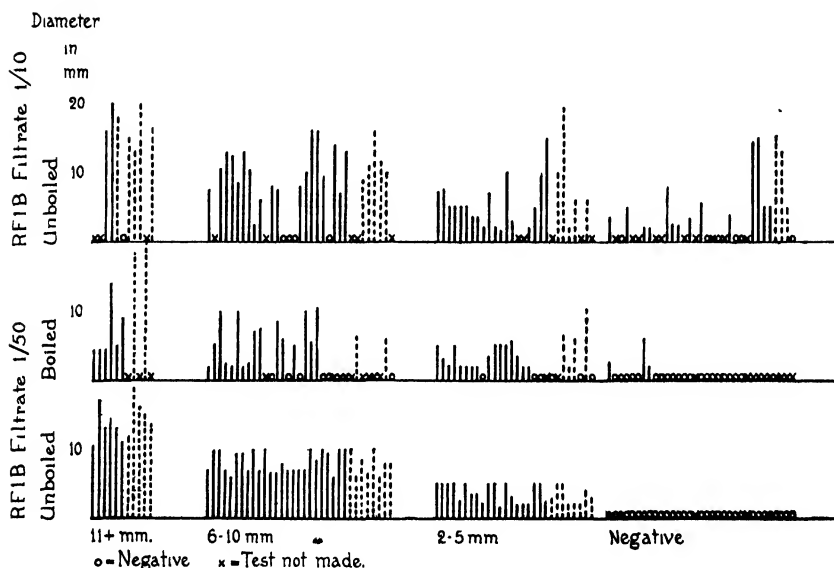


FIG. 2.—Skin reactions with RF1B filtrate in patients with rheumatic fever. O indicates negative and x, test not made.

eliminated, and it was possible to compare accurately the results obtained in patients in different institutions by different observers. The lesions in patients from the wards were measured every twenty-four hours for three days and those in patients from the outpatient clinic only after twenty-four hours, as it was found from the first group that the maximum size was attained in this period.

As many patients in the active stages of the disease were under the influence of drugs, it became desirable to determine their effect on the reactions. Two patients showing lesions with diameters of 14 and 15 mm., respectively, to a 1:100 dilution were given full therapeutic doses of sodium salicylate or neo-cinchophen for three days and then retested; the second reactions were of prac-

tically the same intensity as the first. As other patients under the influence of these drugs seemed to give as strong reactions as would be expected, it was felt

TABLE 1.  
*Reactions on First Test of Patients with Rheumatic Fever.*

Size of Lesions	1 50 Dilution					
	Active Rheumatic Fever		Inactive Rheumatic Fever		Combined	
	No	%	No	%	No	%
11 + mm	5	= 19	6	= 7	11	= 10
From 6 to 10 mm	8	= 31	25	= 28	33	= 28
From 2 to 5 mm	8	= 31	20	= 22	28	= 24
0	5	= 19	39	= 43	44	= 38
Total	26		90		116	
	1 10 Dilution					
	Active Rheumatic Fever		Inactive Rheumatic Fever		Combined	
	No	%	No	%	No	%
11 + mm	12	= 57	15	= 22	27	= 30
From 6 to 10 mm	3	= 14	19	= 28	22	= 24
From 2 to 5 mm	2	= 10	21	= 31	23	= 26
0	4	= 19	13	= 19	17	= 20
Total	21		68		89	
	Controls 1 50 Dilution					
	Other Diseases		No Disease		Combined	
	No	%	No	%	No	%
11 + mm	8	= 20	0	= 0	8	= 13
From 6 to 10 mm	3	= 7	4	= 20	7	= 12
From 2 to 5 mm	5	= 13	5	= 25	10	= 17
0	23	= 60	11	= 55	34	= 58
Total	39		20		59	
	1 10 Dilution					
	Other Diseases		No Disease		Combined	
	No	%	No	%	No	%
11 + mm	10	= 20	5	= 20	15	= 20
From 6 to 10 mm	8	= 16	3	= 6	11	= 15
From 2 to 5 mm	8	= 16	3	= 6	11	= 15
0	23	= 48	15	= 68	38	= 50
Total	49		26		75	

that this medication had little if any influence on the reacting capacity of the patient.

It soon became evident that the best RF1B filtrates furnished us by Dr Birkhaug did not give reactions in nearly so high a proportion of active forms of the

disease as reported by him and Kaiser. In fact, three-fourths of our patients gave negative reactions with 1:100 dilutions; hence it became necessary to use 1:50 and 1:10 dilutions throughout the study. Generally, the test dose was 1:50, with a comparison of 1:10 or 1:100 in many patients. In a number of children, it was impractical to make many injections; hence a complete comparison could not be carried out in all cases. In most instances, a control injection of filtrate boiled for one hour was used. Birkhaug stated in his original communication that inactivation of the filtrate was brought about by this treatment; our observations on this point will be discussed later

TABLE 2.  
*Effect of Heating Filtrate in Various Groups of Patients.*

	Active Rheumatic Fever			Inactive Rheumatic Fever	Nonrheumatic	
	RF1B	P63	RF1B	RF1B	RF1B	RF1B
Dilution .....	1/10	1/10	1/50	1/50	1/50	1/10
Negative .....	0	1	....	19	10	7
Positive, unchanged.	1 = 6%	1 = 10%	1 = 11%	14 = 22%	2 = 12%	0
Positive, became stronger.....	11 = 61%	8 = 80%	6 = 66%	14 = 22%	6 = 35%	3 = 60%
Positive, became weaker .....	6 = 33%	1 = 10%	2 = 22%	35 = 56%	9 = 53%	2 = 40%

### RESULTS.

The reactions obtained on first test of 105 patients with rheumatic fever with 1:50 dilution of filtrate RF1B is given in figure 2; all of these patients were also tested with either boiled filtrate of the same dilution or with unboiled filtrate in 1:10 dilution and most of them with both. The actual average diameter of each lesion is registered; in patients with active disease, the line is broken; in those in whom it was thought to be latent or cured, it is solid. Among this group of patients only 10 per cent gave a reaction of 11 mm. or more in the area tested with the 1:50 dilution, while 31 per cent showed lesions of from 6 to 10 mm. Thus, less than one-half of the entire group gave positive reactions with this dilution. When tested with 1:10 dilutions, about one-third showed lesions of 11 mm. or larger and one-fifth, lesions between 6 and 10 mm. In table 1 are given the figures for these and a few additional cases together with a control group. These

do not include retests, for it seemed that repeated injections increased the patients' sensitiveness in some instances.

It is important to establish some standard as to the size of lesion which shall be considered positive, and this can be done only by comparing the reactions of different groups of persons to various solutions. Some observers think that lesions less than 10 mm. in diameter should not be considered positive. We have, however, made somewhat

TABLE 3.

*Reactions of Patients to Filtrates Heated in Different Manners.\**

	RF1B 1 10				P63 1 10			
	Un-heated	Boiled 1 hour	Boiled 2 hours	Auto-claved 15 minutes	Un-heated	Boiled 1 hour	Boiled 2 hours	Auto-claved 15 minutes
1 R. F.†	7	8	5	j.v.†	6	7	4	j v
2 R. F	14	17	5	19	5	7	5	6
3 R. F	16	22	13	21	10	10	5	4
4 R. F	19	22	15	19	0	0	0	0
5 R. F	18	18	7	9	4	6	6	5
6 R. F	20	22	19	17	6	6	5	5
7 R. F	14	18	5	13	6	11	5	9
8 R. F	11	13	0	11	0	5	6	7
9 Cardiac	0	0	0	0	0	0	0	0
10 Cardionephritic	24	28	22	24	0	0	0	0
11 Normal	22	26	16	18	6	7	4	4
12 Normal	15	17	8	11	7	7	8	9
13 Normal	0	0	0	0	0	0	0	0

\* The figures indicate the diameter in millimeters.

†R. F., rheumatic fever.

‡j.v., just visible.

different classes, as shown in the table. If we include as positive only those of 11 mm. or more, only one-fifth of the patients with active rheumatic fever gave positive reactions with 1:50 dilutions; but nearly two-thirds of them gave positive reactions with 1:10 dilutions. Patients with inactive disease, on the other hand, showed lesions 11 mm. or larger in almost the same proportion as did nonrheumatic controls. If lesions 6 mm. or larger are included as positive, all categories among the patients with rheumatic fever are increased in

considerably higher proportion than among nonrheumatic controls. As only one among twenty-five rheumatic patients showed a lesion of 6 mm. or more with a 1:10 dilution of broth, it seems justifiable to use this figure of 6 mm. as the lower limit of positive reaction, with the reservation that these smaller lesions are designated as only weakly positive. The important point brought out by this part of the study is that patients with active rheumatic fever gave a much higher proportion of positive reactions than did those in whom the disease had become quiescent, and that a considerable number of patients with other diseases also gave positive reactions, although the latter group did not show as high a percentage of positive reactors as occurred among the rheumatic patients.

Reference to figure 2 also shows that patients sometimes gave as strong reactions with 1:50 dilutions as with 1:10 and occasionally gave negative reactions with the stronger dilution but positive reactions with 1:50. Another point is that in the majority of instances the "toxin" was not inactivated by boiling for one hour. The latter point was specially studied in a number of patients who were known to be positive reactors as well as in a number who were tested for the first time. In these patients, the unheated and boiled filtrates were injected on the same day. A summary of the results is shown in table 2.

The interesting point brought out by separating the patients into groups is that in the majority of those with active rheumatic fever the heated filtrate gave stronger reactions than did the unheated, while in those in whom the disease was thought to be inactive and in the nonrheumatic patients the results were almost reversed, and in the larger proportion of cases the heated filtrate gave weaker reactions. Among most of these patients, however, the reaction did not become entirely negative. That increasing the toxicity of the filtrate by boiling was not a peculiar feature of that obtained from RF1B streptococci is shown in the second column of the table; strain P63, a typical *Streptococcus viridans*, with still other strains showed similar increase in toxicity.

A more detailed experiment to determine the effect of heating in various ways was then performed.



One cubic centimeter of RF1B filtrate was placed in each of three glass tubes, which were then sealed by fusing the open ends. One was boiled for one hour in a water bath, the second for two hours and the third was autoclaved at 118 C. for fifteen minutes. Three tubes of P63 filtrate were treated in the same manner. The tubes were then opened, all filtrates were diluted in the proportion of 1:10

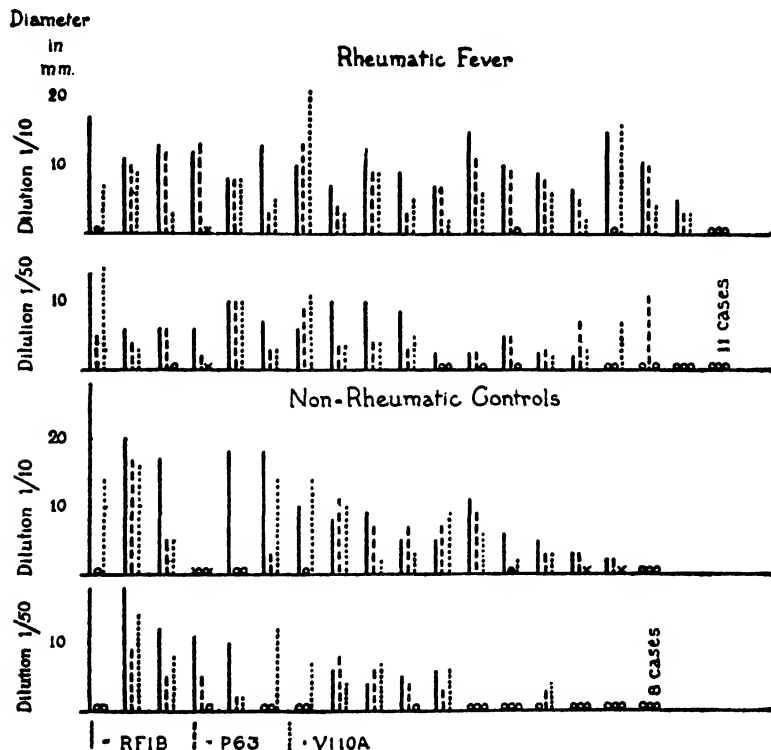


FIG. 3.—Comparison of skin reactions with RF1B, P63 and V110A filtrates. The solid line indicates RF1B; the broken line, P63 and the dotted line, V110A.

with physiologic sodium chloride solution; unheated control filtrate was diluted in the same manner, and thirteen persons were each tested with the eight solutions. The measurements of the lesions twenty-four hours later are shown in table 3.

An interesting immediate reaction was noted in most of the persons tested. This consisted of a rapid enlargement of the wheal produced by the injection of the RF1B filtrate boiled for two hours; in practically all instances, there was a red

aureola from 5 to 20 mm. wide surrounding the wheal. In some of the patients with rheumatic fever, a similar but less marked reaction was noted about the area tested with the P63 filtrate boiled for two hours; but this was practically absent in all of the controls. Thus, it appears that the filtrates, instead of being made less active by boiling, were made more toxic, so that two hours' boiling made them immediately toxic to very sensitive persons such as are found especially in a group of patients suffering from rheumatic fever. In practically all patients, the filtrates boiled for one hour gave larger lesions than did the control filtrates; in most of the patients, those boiled for two hours showed smaller lesions than did the controls at the end of twenty-four hours; the lesions from the autoclaved filtrates were variable. *Streptococcus viridans* P63 filtrate was less active than the RF1B

TABLE 4.  
*Comparison of Lesions Produced by Three Different Filtrates.*

	Rheumatic Fever—All Types			Nonrheumatic		
	RF1B	P63	V110A	RF1B	P63	V110A
1:50 Dilution						
11 + mm.....	1	1	2	4	0	2
From 6 to 10 mm.....	8	5	2	2	3	4
From 2 to 5 mm.....	6	9	8	3	6	3
0.....	14	14	17	15	15	15
No test.....	..	..	..	..	..	..
1:10 Dilution						
11 + mm.....	9	4	2	6	2	4
From 6 to 10 mm.....	8	8	6	4	4	3
From 2 to 5 mm.....	1	5	8	5	5	5
0.....	10	12	10	8	12	9
No test.....	0	0	1	1	1	3

filtrate in all cases. Case 10 is noteworthy: About six weeks before this test the patient, a man, suffered from an acute pyrexia lasting several days, and an intense infiltrated erythema of the thigh; and from his blood, *Streptococcus viridans* was grown in pure culture. After a few days' illness, he recovered and blood cultures were sterile. Thus, a patient known to have suffered a recent infection with *Streptococcus viridans* gave the most marked reactions with filtrate from indifferent streptococci. Case 12 is also noteworthy; the patient, a normal control, is known to be sensitive to filtrates of some hemolytic streptococci, but on a previous occasion gave negative reactions with 1:100 RF1B filtrate. He has, moreover, been repeatedly tested with various hemolytic streptococcus filtrates, and possibly is being made more and more sensitive by this treatment.

Some rabbits which had been made hypersensitive by means of repeated intracutaneous inoculations with *Streptococcus viridans* V110A were found to give well

marked reactions to 1:10 dilutions of RF1B filtrate. Thus, by direct experiment, it was shown that there was a crossing of the hypersensitive state with respect to both green and indifferent streptococci, and that in such hypersensitiveness boiling of the toxic filtrate did not render it inactive. As far as the skin test is applicable, therefore, it seems that the hypersensitiveness of these animals and that of many of the patients tested is comparable.

After the work had progressed a certain distance, it became evident that persons in both the rheumatic fever and control group at times gave positive reactions with filtrates from some strains of *Streptococcus*

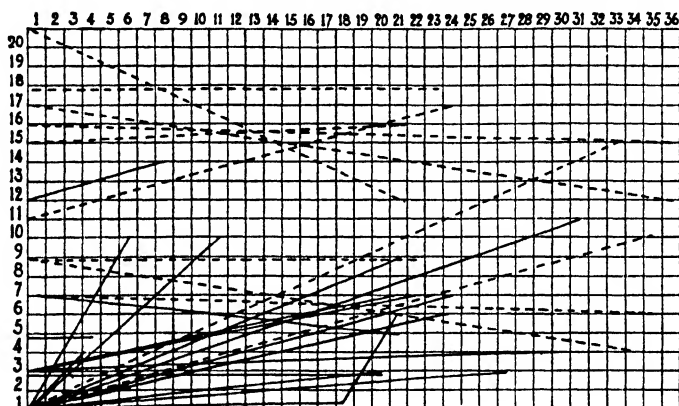


FIG. 4.—Variation in size of skin reactions on repeated tests. The solid line indicates 1:50 dilution, and the broken line, 1:10 dilution of RF1B filtrate.

*viridans*. The most potent strains were P63 and V110A. From them twelve-day filtrates were prepared and a series of comparative tests made with strain RF1B. In most cases, the three unheated filtrates were used in both 1:50 and 1:10 strength. About one-half of the patients had been tested previously. The results are recorded graphically in figure 3, in which the average diameters of the lesions obtained with the three filtrates are placed side by side to render comparison easy. The figures are summarized in table 4. A study of these two shows that many patients who were sensitive to filtrates from indifferent streptococci were also sensitive to filtrates from *Streptococcus viridans*. It is true that the filtrate from the former gave a higher

proportion of positive reactions than from the latter in patients with rheumatic fever, but this was also the case in the nonrheumatic controls. The groups are hardly large enough for statistical comparison; they merely bring out the fact that type specificity in respect of either strain did not exist in either group of persons.

After some patients had been retested two or three times, it was noted that reactions were positive with dilutions of toxic filtrate that previously had given only negative results. In a small series of patients who were retested with either 1:10 or 1:50 dilution of filtrate RF1B, the results are shown in figure 4. The 1:10 dilution is indicated by the broken line and the 1:50 dilution by the solid line. All of these patients were retested within a period of five weeks. The

TABLE 5.  
*Effects of Retesting Within Period of Five Weeks.*

	1:10 Dilution	1:50 Dilution
Increase.....	4	11
Decrease.....	5	1
Same.....	2	2
Always negative.....	3	2
Total.....	13	16

summary of these results, shown in table 5, indicates that in the majority the weaker dilution gave a stronger reaction at the time of second testing than at the first. In eight patients retested after a period of three months or more with this weaker dilution, only three showed a slight increase in strength of reaction. At the time of the first testing most of the patients had received from four to six intracutaneous injections of various dilutions of toxic filtrate. The charted results suggest that patients already somewhat sensitive to such filtrates may at times have their skin sensitiveness increased by these injections. As Birkhaug was able to render himself highly hypersensitive to a filtrate by repeated injections, such a suggestion does not appear unreasonable. These observations indicate at least that for purposes of comparing the skin reactivity of various groups of persons the first test should be the one to be recorded, and a period of from eight to ten weeks should probably elapse before retesting.

The distribution of patients according to age groups is shown in table 6. Unfortunately, the number of patients in the various categories varies so much that comparisons are of little value. Especially is this true when patients with rheumatic fever are compared with nonrheumatic persons. Among the former the proportion of positive reactors increased with advancing years; while among the latter, the figures were more variable, doubtless due to the small number of cases in most groups. To be of value, a study of this point should, however, contain groups of approximately the same size distributed among rheumatic, nonrheumatic and apparently normal persons. It is possible that prolonged infection of the upper respiratory tract, such as tonsillitis or chronic sinusitis, diseases that are

TABLE 6.  
*Proportion of Hypersensitive Individuals in Various Age Groups.*

Age	Rheumatic Fever			Nonrheumatic Fever		
	Number	+	Per Cent	Number	+	Per Cent
3 or under	0	0	0	18	5	28
From 4 to 6	4	0	0	5	3	60
From 7 to 9	20	9	45	7	1	14
From 10 to 12	43	24	56	6	2	33
From 13 to 15	23	13	56	7	1	14
16 +	14	9	64	35	12	34

often encountered in connection with rheumatic fever, may be the factor determining the induction of this type of hypersensitiveness.

It seemed desirable to determine the reaction of a group of patients with rheumatic fever to a filtrate of hemolytic streptococci; and as the toxin from *Streptococcus scarlatinae* has been so extensively used in the Dick test, this was chosen for comparison. Among 112 patients with either active or latent rheumatic fever, 15 showed positive reactions. In 3 others there were negative or faintly positive reactions with unheated toxin but strongly positive reactions with boiled toxin. In 41 patients in whom the Dick test was compared on the same day with their reactions to RF1B filtrate in a dilution of 1:50, the results were as follows: positive to RF1B filtrate and positive to the Dick test,

2; positive to RF1B filtrate and negative to the Dick test, 17; negative to RF1B filtrate and positive to the Dick test, 4; negative to RF1B filtrate and negative to the Dick test, 18. These figures indicate that there was no distinct relationship in the patients' capacity to react to the two types of filtrates. Indeed, the Dick reactions were distributed much the same as would have been expected in any unselected group of children.

#### COMMENT.

While the number of patients tested during this investigation was not so large as that reported by Kaiser, nevertheless it contained representatives of all phases of rheumatic fever and enough controls to indicate at least the significance of these reactions and the type of hypersensitiveness elicited by these tests. There is an additional advantage resting in the fact that they were our own patients and had been studied most carefully from various angles.

The reactions obtained with these filtrates closely resemble those obtained with tuberculin. While there was some local redness within a few hours of the time of injection, the most marked reaction usually occurred about twenty-four hours later. In strong reactors there was distinct brawny infiltration of a dull red and in weak reactors only an intense erythema with little if any palpable infiltration. The filtrate was increased in potency by prolonged incubation of the broth culture. It seemed to be easier to obtain potent filtrates from indifferent streptococci than from green forming strains, although good filtrates were sometimes obtainable from the latter types. As indifferent strains die out much sooner when incubated in broth than do green forming strains and undergo autolysis more readily, it is suggested that the toxic substance is set free from the bodies of the micro-organism as they break up. Mackenzie and Hangar<sup>6</sup> found a "toxin" in certain strains of both green and hemolytic streptococci, but stated that it was rapidly destroyed if the incubation of the culture was continued for forty-eight hours. They also found that many strains rapidly lost their "toxin" producing capacity on repeated subcultures on an artificial medium. In their tests the filtrates were used undiluted; hence their results cannot be closely compared with ours. In one

6. Mackenzie, G. M., and Hangar, F. M., Jr.: *J. Immunol.*, 13: 41, 1927.

respect, however, we agree: the reactions are not strictly specific for rheumatic fever, because when filtrates are used in sufficient strength to give a reaction in the majority of patients with rheumatic fever, they also give reactions in a smaller proportion, but still in a fair number, of patients with other diseases. This is not surprising when we consider the ubiquity of streptococci in the respiratory tract and how frequently they can be recovered from deep in the tonsils, from the paranasal sinuses and from apical abscesses.

Hitchcock<sup>7</sup> has shown that the incidence of indifferent streptococci in the throats of patients with rheumatic fever is no greater than in the throats of other patients. Their ability to produce toxic filtrates seems to rest in some peculiarity of this type of micro-organism rather than in a special specific etiologic rôle in respect of rheumatic fever. It is true that patients with active rheumatic fever show reactions with such filtrates in a greater percentage of instances than do normal persons or persons with other diseases; but this seems to be due to a condition of much increased hypersensitiveness of these patients, for they show a similar hypersensitiveness to filtrates from certain green streptococci. Derick, Hitchcock and one of us (H. F. S.)<sup>8</sup> moreover, have demonstrated marked hypersensitiveness of many of these patients to nucleoproteins prepared from all types of streptococci but most marked to those from hemolytic types. In some patients with weak or negative reactions with filtrates of indifferent streptococci, there were marked reactions with such nucleoproteins.

Another feature of the hypersensitiveness of many patients with rheumatic fever is that when they are tested with boiled filtrate stronger reactions are often elicited than when they are tested with unheated solutions. Most patients with inactive or "cured" rheumatic fever, on the other hand, show less marked reactions with boiled filtrate. While this was not the universal rule in our patients, we were always faced with the difficulty of determining whether or not the disease was inactive. Often there is a low grade persistent infection that permits of correct interpretation only after years of observation. But this difference of reactivity in the two classes of patients with

7. Hitchcock, C. H.: *J. Exper. Med.*, **48**: 403, 1928.

8. Swift, H. F., Derick, C. L., and Hitchcock, C. H.: *Tr. A. Am. Phys.*, 1928, vol. 43, to be published.

rheumatic fever is noteworthy and possibly explains the discordance in the results obtained by us and by Birkhaug and Kaiser. Unfortunately, they did not report the clinical condition of their patients; but it is evident from Kaiser's report that the majority of his patients were in the class designated by us as inactive.

Birkhaug reported that he was able to neutralize the soluble toxin RF1B with an antitoxic serum. He sent us a small amount of this serum; but in a few patients with active rheumatic fever and in some others with strong skin reactions, we were unable to obtain any evidence of neutralization. Some of these patients, on the other hand, showed stronger reaction with the mixture of serum and filtrate than with either alone. We did not carry out enough neutralization experiments to control all phases of the question, but suggest that in this respect there may also be a difference between a patient with active disease and one in the latent stages. Some rabbits which were made hypersensitive to green streptococci and which gave positive reactions with filtrate RF1B also failed to show any neutralization of this toxic filtrate with this antitoxic serum. Mackie and McLachlan<sup>9</sup> sensitized guinea-pigs with living cultures of *Streptococcus scarlatinae* and observed that it was impossible to obtain neutralization of skin reactions with antitoxic serum, and also that heating of the toxic filtrate often did not inactivate it. Some of their animals were also sensitive to filtrates of green streptococci. Zinsser and Grinnell<sup>10</sup> had previously had somewhat similar experiences in regard to sensitization of animals with streptococci from patients with scarlet fever. It is evident from the work of Dochez and Stevens<sup>11</sup> that at least two types of hypersensitiveness to streptococci do exist: one to a toxic product which can be neutralized, and another, probably broader, to a streptococcus product which cannot be neutralized. Because of the number of different strains and products of streptococci to which patients with rheumatic fever react, we are inclined to believe that they have the second type of hypersensitiveness; and that it is the hypersensitiveness of the patient suffering from rheumatic fever rather than the specificity of the toxic filtrate of streptococci which explains

9. Mackie, T. J., and McLachlan, D. G. S.: *Brit. J. Exper. Path.*, **8**: 129, 1927.

10. Zinsser, H., and Grinnell, F. B.: *J. Immunol.*, **10**: 725, 1925.

11. Dochez, A. R., and Stevens, F. A.: *J. Exper. Med.*, **46**: 487, 1927.



the peculiarity of the phenomena observed when these two are brought into contact as in the skin test.<sup>12</sup>

#### SUMMARY.

Patients with rheumatic fever gave a higher proportion of positive skin reactions with filtrates of both indifferent and green streptococci than did nonrheumatic controls.

Patients with active rheumatic fever gave a higher proportion of skin reactions with these filtrates than did those with inactive or "cured" rheumatic fever.

When the reactions with unheated filtrate were compared with those with filtrate boiled one hour it was found that the boiled filtrate gave stronger reactions in most patients with active rheumatic fever and weaker reactions in most patients with inactive or "cured" rheumatic fever.

A close correspondence was not found in the capacity of patients to give skin reactions with Dick "toxin" and filtrates of nonhemolytic streptococci.

Positive reactions in patients with rheumatic fever seem to depend more on the state of hypersensitiveness to certain products of streptococci than on the specificity of any one type of streptococcus.

12. See also Strauss, H.: *Deutsche med. Wchnschr.*, **53**: 737, 1927.



## VARIANTS OF HEMOLYTIC STREPTOCOCCI; THEIR RELATION TO TYPE-SPECIFIC SUBSTANCE, VIRULENCE, AND TOXIN.

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In previous communications one of us (1) described three substances which can be extracted from hemolytic streptococci: (1) *The nucleoprotein P* is common to all strains of hemolytic streptococci and is serologically related to the nucleoproteins of pneumococci and of green streptococci. (2) *The non-protein substance C*, which appears to be a carbohydrate, is found in all strains of hemolytic streptococci but is species-specific and serologically distinct from the carbohydrate fractions of pneumococci and green streptococci. (3) *The type-specific fraction M*, which is probably protein in nature, has not been isolated from other species of microorganisms; it occurs in serologically distinguishable forms which serve to differentiate hemolytic streptococci into types.

One of us (2) has previously described two forms of hemolytic streptococci distinguishable by the morphology of their colonies. The general appearances of these colonies, when grown on a special medium and viewed by reflected light, are the same as those which distinguish the rough and smooth varieties of other bacteria but the terms "R" and "S" have not been used and the colonies have been designated "matt" and "glossy" to avoid confusion which would otherwise certainly arise from the circumstance that the rough, or matt, colonies are the virulent type while the smooth, or glossy, forms are relatively avirulent. It is the purpose of this paper to show that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies.

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It will also be shown that filtrates from both matt and glossy cultures of hemolytic streptococci contain skin-reactive toxin.

*Some Characteristics of the Type-Specific Substance M.*

The type-specific substance M is prepared by a modification of Porges' (3) method. The bacterial bodies are extracted with N/20 HCl in salt solution at the temperature of boiling water; and after neutralization and clarification by centrifuging the resulting clear slightly yellow fluid is used as an antigen for precipitin reactions. Bacterial extracts prepared in this manner contain the type-specific substance M and in addition they also contain small quantities of the non-type-specific fractions, P and C, which may cause some precipitation with sera prepared against any strain of hemolytic streptococcus. To avoid the appearance of these confusing precipitates the serum may be absorbed with any heterologous strain of hemolytic streptococcus and by this means a specific anti-M serum is obtained which will only precipitate in the presence of the homologous M substance.

In the original work (1) which led to the recognition of the type-specific substance M thirteen strains were used which Dochez, Avery and Lancefield (4) had classified, some years earlier, into types by agglutination and protection tests. Ten of these strains yielded type-specific precipitating substances which differentiated them into types corresponding to those originally determined. The remaining three strains failed to yield any type-specific substance although they had been classified by agglutination and protection in the earlier work. It was suggested that prolonged cultivation in the laboratory had caused these three strains to lose their type-specific characteristics. In the present communication it will be shown that matt cultures containing the type-specific substance can, by various means, be reduced to the glossy form in which the type-specific substance is no longer present.

*The Preparation of Anti-M Serum.*

As the type-specific substance M, after separation from the bacterial cell, does not produce any demonstrable antibody, when injected into animals, the only antigen available for preparing anti-M serum

is a suspension of bacteria in the matt form. Matt cocci contain the three substances P, C and M and sera prepared with these organisms, therefore, contain antibodies to each of the three substances. On the other hand, cocci in the glossy form contain the two substances P and C but are devoid of the type-specific substance M; consequently, antibacterial sera prepared with glossy strains contain antibodies to P and C but do not contain any type-specific antibody.

Anti-M sera were prepared by inoculating rabbits intravenously with 16 hour cultures of matt cocci grown in tryptic broth. Immunization was commenced with four injections on consecutive days of 1 cc. of heat-killed culture, followed a week later by four doses of 2 cc. of the same vaccine. During the 3rd week the rabbits received four doses of 0.5 cc. of living culture and in the 4th week this dose was doubled. A final series of doses of 2 cc. of living culture was given during the 5th week and the animals were bled 10 days after the last injection. The sera of animals immunized in this way usually contained a satisfactory quantity of antibody to the type-specific substance M but with some strains it was necessary to continue immunization with 5 cc. and even 10 cc. of living culture before useful sera could be secured. A few strains have been encountered which, although they were in the matt form and moderately virulent for mice (0.001 cc. or 0.0001 cc.), produced only traces of type-specific antibody in rabbits even after intensive immunization. Twelve rats were immunized with a strain (New York V E14) which had previously failed to produce more than traces of type-specific antibody in the sera of twelve immunized rabbits. The rats, which remained perfectly well during immunization, received the following intraperitoneal doses: 1st week 3 doses of 0.25 cc. of heat-killed culture; 2nd week 4 doses of 0.5 cc. of heat-killed culture; 3rd week 4 doses of 1.0 cc. of heat-killed culture; 4th week 3 doses of 1.0 cc. of living culture; 5th week 4 doses of 2.0 cc. of living culture; 6th week 4 doses of 2.0 cc. of living culture. Seven of the immunized rats yielded moderately good anti-M sera; the remaining five sera contained traces of type-specific antibody. As rats appear to be able to tolerate relatively larger doses of culture than rabbits it is possible that they may be more suitable for the preparation of anti-M serum; but the small yield of serum makes this method impracticable for routine purposes.

#### *The Absence of the Type-Specific Substance M from Organisms Which Form Glossy Colonies.*

It has already been stated that the type-specific substance M is found in HCl extracts of matt hemolytic streptococci and that it is not found in similar extracts prepared from the glossy variants.

This is demonstrated by the following experiment:

Four type-specific anti-M sera were prepared by immunizing rabbits with four matt strains of hemolytic streptococci belonging to different serological types and by subsequently removing the non-type-specific antibodies from the sera by absorption with heterologous strains.

Table I gives the precipitin reactions of the four sera with HCl extracts prepared from cultures of the homologous cocci (1) in the matt form and (2) in the glossy variant form.

TABLE I.

*Precipitin Reactions of Type-Specific Anti-M Sera with Extracts of the Homologous Strains (1) in the Matt Form, (2) in the Glossy Form.*

Volumes of extracts*	Strain S43		Strain S23		Strain C203		Strain London	
	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form
cc.								
0.4	+++	—	++++	—	++	±	++	—
0.1	++	—	++	—	++±	—	++±	—
0.025	+	—	+	—	++	—	±	—

\* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

It will be seen from Table I that the extract prepared from the matt form of each strain gave a good precipitin reaction with the homologous antiserum; on the other hand, the extract prepared from the glossy form of each strain gave a negative precipitin reaction with the single exception of Strain C203 which gave a faintly positive reaction. Although this experiment seems to show that each of the four strains lost its type-specific substance in the process of degradation to the glossy variant form, yet it will be seen from the results of experiments with highly concentrated extracts that, in reality, only one strain had completely lost its type-specific substance. Highly concentrated extracts were prepared from each of the four glossy variants referred to in Table I in the following manner: The centrifuged deposit from 9 liters of broth culture was extracted with HCl; and, after concentration by alcoholic precipitation, the precipitate was redissolved in

5 cc. of salt solution. Precipitin tests, with the concentrated extract prepared from Strain S23 were negative showing that this strain was completely devoid of type-specific substance. Precipitin tests, with the other three concentrated extracts and their homologous specific anti-M sera, were weakly positive showing that three of the glossy cultures retained traces of type-specific substance. The minute amounts of specific substance remaining in these cultures can be judged from the following figures—9 liters of broth culture were used in preparing extracts from the glossy forms—50 cc. of broth culture were used in preparing extracts from the matt forms—180 times more culture was, therefore, used in the preparation of the glossy extracts than in the preparation of the matt extracts and, in spite of these disproportionate quantities, the latter extracts contained the larger quantity of the type-specific substance M. It appears from these experiments that hemolytic streptococci are rarely degraded to the point at which type-specific substance completely disappears.

*Some Characteristics of Matt Cultures of Hemolytic Streptococci.*

Twenty-eight strains of hemolytic streptococci were examined immediately after isolation from pathological conditions in the human body. The sources of these cultures included cases of puerperal septicemia, pleural effusion, scarlet fever, pneumonia and sinusitis; strains were also isolated from the depths of enucleated tonsils and from throat swabs. In twenty-one cases the cultures when freshly isolated, were entirely composed of matt colonies; in five cases both matt and glossy colonies were seen on the plates; and in two cases the cultures were entirely glossy, but as both the glossy strains were obtained from throat swabs and were accompanied by other bacteria there was no evidence that the hemolytic streptococci were playing a pathogenic rôle. Table II gives the source and character of the cultures.

There is, therefore, some evidence that cultures freshly isolated from human sources are usually of the matt variety and this statement particularly applies to diseases such as septicemia in which the streptococci are the undoubted causal agent.

It is frequently found that matt strains of hemolytic streptococci, isolated from human lesions and undoubtedly pathogenic for man, are

TABLE II.  
*The Morphological Appearance of Colonies of Freshly Isolated Strains of Hemolytic Streptococci and the Source of the Cultures.*

No.	Disease	Source of culture	Pure culture or mixed flora	Morphology of colonies
1	Puerperal septicemia	Blood culture	Pure culture	Matt
2	"	"	"	"
3	"	"	"	"
4	Nephritis and septicemia	"	"	"
5	Endocarditis	Postmortem culture from spleen	"	"
6	Pneumonia and pleural effusion	Chest fluid	"	"
7	Sinusitis	Nasal mucus	"	"
8	Scarlet fever	Throat swab	Almost pure culture	"
9	"	"	"	"
10	Tonsillitis	"	"	"
11	Enlarged tonsils	From depths of enucleated tonsils	"	"
12	"	"	"	"
13	"	"	"	"
14	"	"	"	"
15	Pharyngitis	Swab from pharynx	"	"
16	Sinusitis	" " nose	"	"
17	Tonsillitis	" " tonsils	"	"
18	"	" " "	"	"
19	Pneumonia	Sputum	Chiefly <i>S. hemolyticus</i> ; <i>B. influenzae</i>	"
20	"	"	Mixed flora	"
21	" (Type IV pneumococcus)	"	Equal numbers hemolytic streptococci and pneumococci	"



22	Pneumonia (Type IV pneumococcus)	Sputum From depths of tonsils	Mixed flora Pure culture	Matt and glossy " " "
23	Enlarged tonsils			
24	"			
25	"			
26	"	"	Almost pure culture " " " "	" " " " " " " "
27	Tonsillitis	Swab from tonsils	Mixed flora, about 5 per cent hemolytic streptococci	Glossy
28	Pneumonia (Type IV pneumococcus)	" " "	Mixed flora	"

avirulent for mice (M.L.D. 0.5 cc. or 1.0 cc.). Such a culture entirely composed of matt colonies will be referred to in this paper as the matt attenuated form because it is avirulent for mice yet possesses the colony characteristics and the specific substance of the virulent form. Attempts to increase the virulence of matt attenuated cultures, by mouse passage, have always been successful although some of the strains tested have required very many passages before the maximal virulence of 0.000001 cc. has been attained and in some cases the virulence has never risen above 0.0001 cc. even after 80 or 90 consecutive passages through mice.

Virulence is the only quality which distinguishes the matt virulent form from the matt attenuated variety as these colonies are identical in appearance, and serological examination of HCl extracts does not show any significant difference in the quantity of type-specific substance which can be extracted from equal volumes of the two cultures. From these experiments it appears that the matt form, which is always potentially virulent, may occur in, at least, two separate varieties characterized by quantitative differences in virulence for mice; and it is probable that, by suitable passage experiments, additional forms can be obtained distinguishable by different degrees of virulence for other species of animals.

*Methods of Converting the Matt Form to the Glossy Variant.*

The degree of ease with which glossy variants can be obtained from different strains varies enormously. In some cases great difficulty is experienced in maintaining laboratory stock cultures of the matt, or potentially virulent, form as they spontaneously change to the glossy variant even when stored in blood broth in the ice box. In these cases it is necessary to resort to frequent mouse passages to prevent the total loss of the matt form. On the other hand, matt strains have been encountered which do not show any tendency to change to the glossy variant after repeated subcultivations on agar. Intermediate between these extremes are strains which develop a small proportion of glossy colonies after repeated subcultivations on agar. When one of the glossy colonies derived from these strains is subcultured in broth and replated on agar a mixture of matt and glossy colonies usually appears but occasionally a pure glossy culture

may be obtained by this method. By repeated selection of glossy colonies and subcultivation in broth a culture composed entirely of glossy colonies can often be obtained and in some instances a pure culture of the glossy variant can be secured by the simple process of repeated subcultivation on agar slants.

Griffith (5) and others (6-8) have shown that smooth pneumococci can be converted to the rough form by cultivation in the homologous anti-S serum. We have applied this technique to hemolytic streptococci and have found that the cultivation of matt strains in undiluted homologous anti-M serum of high titer is the quickest and most reliable method of obtaining glossy variants, and with some highly stable matt strains this is the only method by which we have been able to secure the glossy form. Here again there are wide differences between individual strains; in some cases, a few transfers in serum suffice to convert a virulent matt culture, containing abundant M substance, into the glossy avirulent variant devoid of any specific substance; in other cases, after as many as 90 transfers in high titer serum traces of the type-specific substance M can still be detected in concentrated bacterial extracts, although the colonies appear to be glossy and the organisms have lost their virulence for mice. Attempts have been made to rid these cultures of the remaining traces of type-specific substance by alternately cultivating the cocci in immune serum, plating out, selecting the most glossy colonies and again subculturing in immune serum; one strain was subjected to this treatment thirty times after 90 previous consecutive transfers in immune serum but at the end of the experiment it still retained traces of the specific substance.

#### *Some Characteristics of Glossy Cultures of Hemolytic Streptococci.*

The glossy variant is avirulent for mice in comparison with the matt virulent culture from which it is derived and attempts to raise the virulence of the variants by mouse passage have usually been unsuccessful. It is, however, possible to obtain glossy cultures which are partially virulent for mice. A strain which, in the matt virulent form, killed mice regularly in doses of 0.000001 cc. or 0.0000001 cc. was cultivated in the homologous anti-M serum and, after 55 transfers in 50 per cent serum, a pure culture of the glossy variant was ob-

tained which did not contain any type-specific substance, yet this glossy culture was sufficiently virulent to kill mice regularly in doses of 0.01 cc. and occasionally in doses of 0.001 cc. More prolonged cultivation in anti-M serum did not cause any further decrease in virulence. The partially virulent glossy culture was passed through twenty-five mice intraperitoneally but the virulence remained unchanged and there was no reappearance of type-specific substance. This appears to be an exceptional strain as the M.L.D. of the majority of glossy strains is 0.5 cc. or 1.0 cc.

During the process of conversion from matt to glossy various types of colonies appear, which may possibly represent intermediate forms or may be due to individual colonies containing a mixture of matt and glossy cocci. We have observed that different strains sometimes show peculiarities in the morphology of their colonies which are so striking that the strain can be recognized either in the matt or glossy form. In addition to these strain peculiarities other varieties of colonies appear during the gradual change from matt to glossy. Griffith (9) has noted that in spite of the apparently diverse appearances of streptococcal colonies three forms can generally be distinguished. Two of his forms appear to correspond to our matt and glossy colonies and the third is characterized by a soft consistency, a whitish opaque raised center and a thin translucent margin. In a previous communication one of us (10) described a similar form of colony, which differed in the important respect of being tough instead of watery; but further observation has shown that colonies characterized by a flat marginal zone surrounding a central eminence may occur in two forms, corresponding to the matt or to the glossy state. The matt form of this colony is opaque and of tough consistency with a central eminence surrounded by a flat marginal zone; the glossy form has a similar contour but is soft and watery. The irregular shape of these colonies causes difficulty in observing the light-reflecting character of their surfaces but the matt and glossy forms can generally be distinguished by other characteristics. These observations seem to indicate that this third type of colony is not a distinct entity separate from the matt and glossy forms; but we have failed to determine the significance of these very characteristic colonies.

The classification of colonies is further complicated by the occasional appearance of pseudoglossy forms. When a matt culture is spread on a plate the colonies in close proximity to each other may present the typical matt appearance but widely separated colonies in the same culture may be glossy in appearance. If one of the latter colonies is selected and spread on a fresh plate a pure culture of typical matt colonies may result. Pseudoglossy colonies are generally larger than true matt or true glossy colonies but they so nearly resemble the true glossy form that they are liable to cause confusion.

Owing to these variations in the appearance of colonies we have been unable to rely entirely on the colony form as a guide to the character of cultures. The criteria we have used to determine when a culture is completely degraded from the matt state are: (1) that concentrated HCl extracts of glossy cultures should not cause any precipitation when mixed with pure homologous anti-M serum (absorbed with a heterologous strain to remove the antibodies to P and C); (2) that the result of the above test should remain unchanged after the culture has been passed through a mouse.

It will be seen in the detailed description of experiments that we have only been able to secure one strain in this completely degraded state.

#### *Reversion of Glossy Cultures to the Matt Form.*

Dawson and Avery (11) have shown that many strains of R pneumococci can be reverted to the S form by repeated mouse passages or by cultivation *in vitro* in anti-R serum. Griffith (12) has shown that R pneumococci frequently revert when they are mixed with large doses of heat-killed S pneumococci and inoculated subcutaneously into mice.

We have attempted to revert glossy hemolytic streptococci to the matt form by each of these three methods.

*1. Mouse Passage.*—Passage experiments have been done with glossy cultures derived from five different strains (S3, S23, Henson, S43, C203) but no definite evidence has been obtained that reversion can be achieved by this method. In two cases, S3 (ten passages) and Henson (twenty-five passages), the glossy character of the cultures was judged entirely by the appearance of the colonies as no anti-M serum was available for these strains.

The virulence (0.1 cc.) and colony form of Strain S3 remained unchanged after passage through ten mice.

Passage of Strain Henson through twenty-five mice caused the virulence of the culture to rise from 0.5 cc. to 0.01 cc. and this change was accompanied by a slight alteration in colony form, many of the colonies in the passage culture having flat tops instead of the typical dome-shaped appearance of glossy colonies. In this instance the partial restoration of virulence and the accompanying change in colony structure may possibly indicate that reversion had commenced but the evidence is inconclusive in the absence of serological proof that the culture had been completely degraded before the mouse passages were commenced.

The glossy culture of Strain S23 appeared to be completely degraded as no trace of precipitate was formed when highly concentrated HCl extracts were mixed with the homologous type-specific antibody. This culture was passed through twenty-seven mice and at the end of the experiment the virulence for mice (0.01 cc.) and the colony form remained unchanged and there was no re-appearance of type-specific substance.

The glossy culture of Strain S43 was not completely degraded as, although unconcentrated extracts of the variant culture did not precipitate the homologous anti-M serum, yet traces of type-specific substance could be demonstrated in concentrated HCl extracts. This culture was passed through a series of mice and examination of unconcentrated extracts, after each passage, showed the gradual reappearance of type-specific substance so that after eight passages the culture was equal to the original matt form in its yield of type-specific substance. The virulence (0.1 cc.) and colony form were unaltered by eight passages but this experiment seems to indicate that a culture which has lost the major part of its type-specific substance and yet retained a fraction of its original specificity can be reverted to the original form with comparative ease.

The variant culture of Strain C203 formed typical glossy colonies but concentrated HCl extracts of the cocci contained traces of type-specific substance. After passage through ten mice the virulence (0.1 cc.) and the colony form of the culture remained unchanged and there was no increase in the quantity of type-specific substance which could be extracted from the cocci.

2. *Cultivation in Immune Serum.*—A glossy culture of Strain New York V was obtained which failed to kill mice in a dose of 0.5 cc. Rabbits were immunized with this culture and a serum was obtained which agglutinated the glossy culture up to a dilution of 1 in 2,560. The glossy culture was grown in various dilutions of this serum (5 per cent, 10 per cent, 50 per cent, 100 per cent) for a number of transfers but in no case was there any evidence of reversion—50 per cent serum was selected as the concentration in which the cocci appeared to multiply most freely and the culture was carried 118 transfers in this medium. At the end of the experiment the form of the colonies and the virulence of the culture remained unchanged.

Immune serum prepared against glossy cocci contains antibodies to the two non-type-specific fractions, P and C. In the following experiment the influence

of pure anti-P serum on glossy cultures of four strains was tested. High titer anti-P serum was prepared by immunizing rabbits with purified nucleoprotein extracted from hemolytic streptococci. The glossy variant forms of four strains (S43, S23, C203, London) were cultivated for twelve transfers in a 10 per cent dilution of this serum. This treatment did not alter the colony forms of the cultures although the virulence of two strains (S23 and London) was definitely increased. The quantities of type-specific substance, however, which could be extracted from the cocci of all four strains remained unchanged.

3. *Subcutaneous Inoculation of Mice with Glossy Cultures in Combination with the Homologous Matt Cocci Killed by Heat.*—A few experiments have been done with one of our glossy strains (Henson) in an attempt to revert this culture to the matt form by the technique devised by Griffith for the reversion of R pneumococci to the S form. A heavy suspension of the matt culture was prepared by heating, at 60°C. for 30 minutes, the deposit from 50 cc. of culture, concentrated to 2 cc. 0.5 cc. of the heat-killed suspension was mixed with 0.05 cc. of living glossy culture and injected subcutaneously into a mouse. Cultures from the lesion in the mouse contained both matt and glossy colonies although controls indicated that the matt organisms of the heated suspension were dead. Unfortunately, this technique, so successful with pneumococci, is not altogether satisfactory for hemolytic streptococci as it has been found impossible to avoid ulceration when large numbers of heat-killed matt organisms are combined with glossy cultures and even small doses of glossy culture alone frequently cause ulceration. Matt colonies isolated from these open ulcers must be viewed with suspicion since they may arise from contaminating cocci, but it seems probable that further work with this technique may yield convincing evidence of the reversion of the glossy cocci to the matt form.

So far as any conclusions can be drawn from the limited number of observations recorded it seems that the glossy variant is a highly stable form but that reversion may occur under certain conditions.

#### *A Comparison of the Toxigenicity of Matt and Glossy Cultures of the Same Strain.*

The method used for comparing the toxigenicity, the virulence for mice and the colony appearance of matt and glossy cultures of the same strain was as follows:

Young broth cultures of the different forms of each strain were sown in 50 cc. of tryptic digest broth. After 16 hours incubation the virulence and colony appearance of a sample taken from each culture were determined; and the flasks were then returned to the incubator. The cultures were filtered after 4 days of incubation and the filtrates were tested by injecting 0.1 cc. of diluted filtrate into

TABLE III.  
A Comparison of the Toxicogenicity and Virulence of Matt and Glossy Cultures of the Same Strain.

	Identification number of patient	Filtrate from matt virulent culture	Filtrate from matt attenuated culture	Filtrate from glossy culture
<i>1. Broncho pneumonia strains</i>				
Strain S43.	Active filtrate diluted 1 in 500	15 mm. (10 <sup>-7</sup> )	15 mm. (10 <sup>-1</sup> )	13 mm. (10 <sup>-1</sup> )
"	" " " 1 " 500	11 "	10 "	8 "
"	" " " 1 " 50	27 "	23 "	26 "
"	" " " 1 " 50	18 "	9 "	14 "
"	" " " 1 " 100	22 " (10 <sup>-7</sup> )	—	20 " (10 <sup>-7</sup> )
"	" " " 1 " 100	6 "	—	11 "
<i>2. Scarlet fever strains</i>				
Strain C203.	Active filtrate diluted 1 in 1,000	28 " (10 <sup>-7</sup> )	—	20 " (less than 10 <sup>-1</sup> )
"	" " " 1 " 1,000	2 "	—	7 "
"	" " " 1 " 1,000	18 " (10 <sup>-7</sup> )	—	19 " (10 <sup>-1</sup> )
"	" " " 1 " 1,000	0	—	0
"	" " " 1 " 1,000	26 mm. (10 <sup>-7</sup> )	—	32 mm. (10 <sup>-1</sup> )
"	" " " 1 " 1,000	0	—	0
"	" " " 1 " 1,000	10 mm. (10 <sup>-7</sup> )	—	10 mm. (10 <sup>-1</sup> )
"	" " " 1 " 1,000	0	—	0
"	" " " 1 " 1,000	11 mm. (10 <sup>-7</sup> )	—	18 mm. (10 <sup>-1</sup> )
"	" " " 1 " 1,000	0	—	0

The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared. 0 indicates no reaction; — indicates not tested.



the skin of one, or more, known positive reactors. Table III gives the dimensions of the reactions which followed the injection of active filtrate and of the same filtrate after heating in boiling water for 2 hours. Measurements, which are given in mm. representing the average diameter of the skin reactions, were taken 24 hours after injection. The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.

The two strains isolated from cases of bronchopneumonia produced weak toxic filtrates in comparison with the scarlet fever strains and were therefore used in greater concentration.

It will be seen from Table III that the filtrates from the different forms of each strain caused approximately equal reactions and that no correlation could be established between virulence and toxigenicity.

#### DISCUSSION.

It may be stated as a broad generalization that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies. This is analogous to the invariable presence of the soluble specific substance S in virulent cultures of pneumococci and its absence from avirulent R cultures, but here the analogy breaks down as far as virulence for mice is concerned, since it is possible to prepare matt cultures of hemolytic streptococci which contain large quantities of the type-specific substance M and yet are avirulent for mice.

One of the most striking characteristics of hemolytic streptococci is the difficulty which has always been experienced in securing highly virulent cultures of a large number of strains. This is undoubtedly due in part to the fact that the glossy variant is a highly stable avirulent form but even when we exclude this variant form and confine our attention to the matt or potentially virulent varieties we are still unable to secure highly virulent cultures with any degree of ease. The behavior of matt attenuated cultures undergoing mouse passage is frequently capricious; virulence generally rises to a moderate level after the initial ten, or twenty passages and it may then increase suddenly, or it may gradually increase after many more passages, or it may remain for an indefinite period in a state of mediocrity. This is in contrast to pneumococci which appear to be either rough and avirulent for mice or smooth and of maximal virulence for this

species. The virulence of pneumococci appears to be intimately associated with the presence or absence of the soluble specific substance S; in the case of hemolytic streptococci, however, virulence is not entirely dependent on the presence or absence of the type-specific substance M; some additional unknown factor is operative. Glossy variants, when fully degraded, contain no type-specific substance and are avirulent for mice; matt organisms occur in two forms equally rich in type-specific substance—one of these forms is no more virulent for mice than the glossy variant, the other is highly virulent.

It is possible that this contrast between pneumococci and hemolytic streptococci may be partly due to differences in bacterial structure. In the case of pneumococci the soluble specific substance S is disposed in a capsular layer over the surface of the organism; but microscopic examination of hemolytic streptococci gives no information as to the situation of the type-specific substance M in the bacterial bodies; and it is possible that the distribution of this substance throughout the organisms may render it less accessible and therefore less susceptible to external influences.

Certain strains of hemolytic streptococci exhibit an unexpected stability in all forms. Cultures which are partially degraded so that they contain only small quantities of type-specific substance may be passed through a number of mice without any accumulation of type-specific substance and without any alteration of virulence. Conversely, when the virulence of a matt strain has become established it is difficult to reduce the culture to the matt attenuated state and at the same time to avoid conversion to the glossy variant form.

No relationship could be established between toxigenicity and virulence; in some instances highly virulent matt cultures produced weak toxic filtrates and the glossy variant avirulent forms were equally toxigenic; in other instances relatively avirulent matt strains produced highly toxic filtrates.

It appears therefore that virulence is not determined by toxigenicity and is not entirely dependent on the presence or absence of type-specific substance although cultures which have lost their type specificity are invariably avirulent.

An unknown factor determines whether hemolytic streptococci,

which contain their full quota of type-specific substance, are virulent or attenuated.

#### SUMMARY.

Hemolytic streptococci, when freshly isolated from pathogenic lesions, form characteristic matt colonies and contain the type-specific substance M.

Two varieties of matt cultures, equally rich in type-specific substance, can be distinguished by the virulence of the organisms for mice: (1) the matt virulent variety, (2) the matt attenuated variety.

The matt forms of hemolytic streptococci can be degraded to a third variety which forms glossy colonies and is always relatively avirulent. This is accomplished by prolonged cultivation on artificial media, by selection of colonies or by cultivation in homologous anti-M serum. In the process of degradation the cocci lose the major part of their type-specific substance but complete disappearance of type-specific substance rarely occurs.

The glossy variant form, when fully degraded, is highly stable; but glossy cultures which have retained some type-specific substance can occasionally be reverted to the original matt form.

Toxic filtrates from matt and glossy cultures are approximately equal in skin reactivity.

No relationship appears to exist between virulence and toxigenicity.

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## ANTIGENIC DIFFERENCES BETWEEN MATT HEMOLYTIC STREPTOCOCCI AND THEIR GLOSSY VARIANTS.

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In a previous communication (1) it was shown that hemolytic streptococci in the matt form contain the type-specific substance M and that when matt cultures are degraded to the glossy variant form the organisms lose their type-specific substance. The antigenic differences between matt and glossy cultures, which are described in the present paper, were demonstrated with four different strains of hemolytic streptococci.

Owing to individual differences between the four strains it is essential to describe each series of experiments separately.

### A. Strain S43.

This strain was isolated from a case of bronchopneumonia and had been kept in stock cultures for 10 years before our work was commenced. In spite of this long interval, however, the culture was entirely composed of matt colonies. Virulence tests proved that 0.1 cc. killed mice regularly in 24 hours but mice receiving 0.01 cc. survived indefinitely.<sup>1</sup>

The culture was, therefore, in the matt attenuated state and efforts were made to obtain the matt virulent form and the glossy variant so

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<sup>1</sup> A standard technique was used in all the virulence tests recorded in this paper. Cultures were sown in tryptic digest broth and incubated for 16 hours. Tenfold dilutions of culture, in infusion broth, were injected intraperitoneally into a series of white mice, each mouse receiving 0.5 cc. of the appropriate dilution. In some cases counts were made by plating dilutions in blood agar and although this was not done as a routine in every test, a sufficient number of observations were made to show that the dose representing 0.000001 cc. of culture usually contained about 100 organisms.

that the three forms of the same strain could be compared and their antigenic relationships established.

The matt virulent form was secured by passing the original culture through a series of mice (0.1 cc. or 0.2 cc. of peritoneal washings being transferred directly from mouse to mouse), and, although there was an initial increase of virulence, after the first few passages, it required 51 passages before 0.000001 cc. of culture could be relied upon to kill mice regularly.

It was even more difficult to obtain the glossy variant from this highly stable matt strain, as prolonged subcultivation on agar slants did not alter the typical

TABLE I.

*Precipitin Reactions of Extracts of S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy with the Homologous Pure Anti-M Serum from Which Non-Type-Specific Antibodies Had Been Removed by Absorption with a Heterologous Strain.*

Anti-M serum absorbed with hetero- logous strain*	Volume of extracts†	Precipitates with extracts from		
		S43 matt virulent	S43 matt attenuated	S43 glossy
0.2 cc.	0.3 cc.	+++	+++	—
0.2 cc.	0.1 cc.	++	++	—
0.2 cc.	0.025 cc.	+	+	—

\* Serum R166, against Strain S43 matt attenuated.

† These volumes of crude HCl extract were made up to 0.3 cc. with saline.

The tests were incubated 2 hours at 37°C. and read after standing in the ice box overnight.

+++ , ++ , + represent degrees of precipitate.

— represents no precipitate.

matt appearance of the colonies. The original culture was, therefore, grown in undiluted, high titer anti-M serum, prepared by immunizing rabbits with the matt attenuated form, and after sixteen transfers in this medium the appearance of the colonies seemed to indicate that the culture had been reduced to the glossy state.<sup>2</sup> Table I shows the precipitin reactions of HCl extracts prepared from the three forms of culture with pure anti-M serum previously absorbed with a heterologous strain to remove antibodies to P and C.

Extracts of the matt virulent and of the matt attenuated cultures formed equally heavy precipitates with anti-M serum but a similar extract of the glossy

<sup>2</sup> Strain S43 in the matt state formed typical matt colonies, but colonies of the variant culture were characterized by a heaped up center surrounded by a wide flat margin and they never assumed the typical glossy appearance.

culture did not cause any precipitation.<sup>3</sup> This experiment showed that the variant contained little, if any, type-specific substance but subsequent examination of a concentrated extract proved that the culture was not entirely free from the type-specific fraction. An HCl extract was made from the deposit of 9 liters of culture, it was then concentrated by precipitation with alcohol and redissolved in 5 cc. of saline. This concentrated and purified extract caused some precipitation with the pure anti-M serum showing that the culture still retained traces of type-specific substance. Further attempts to reduce this strain to a completely degraded state were unsuccessful; colony selection was combined with more than 120 transfers in immune serum but even after this treatment traces of type-specific substance could still be detected.

Three kinds of antiserum were prepared by immunizing three groups of rabbits with the three forms of Strain S43. Four rabbits were included in each group and all received equal doses of the appropriate cultures on the same days—the whole series being finally bled on the same day. The immune sera were used for precipitin reactions and for mouse protection tests; in the latter tests, 0.5 cc. of serum was injected into the peritoneal cavity of a series of mice and the degree of passive immunity conferred was tested, on the following day, by inoculating the mice intraperitoneally with graduated doses of virulent culture.

*Precipitin Tests.*—Table II gives the results of precipitin reactions with the three kinds of sera and purified solutions of the nucleoprotein P, the carbohydrate C and the homologous type-specific substance M.

The non-type-specific fractions P and C precipitated the three kinds of sera to a similar extent though, as usual, the anti-glossy sera contained, in the aggregate, an excess of C antibody. The type-specific substance M, in contrast to the P and C fractions, formed heavy flocculent precipitates with the anti-matt sera but only traces of precipitate with the anti-glossy sera. The appearance of these traces of precipitate confirmed the observation, already noted above, that the S43 glossy form was not completely degraded by showing that it

<sup>3</sup> Crude HCl extracts of glossy cultures when fully degraded, are free from type-specific substance but they contain the non-type-specific fractions P and C and will consequently precipitate *unabsorbed* antibacterial serum prepared against any strain of hemolytic streptococcus. Precipitates due to the C fraction are easily recognized as they appear late and form compact discs quite unlike the flocculent precipitates of the type-specific fraction which begin to appear as soon as the extract comes in contact with the serum. Precipitates due to the nucleoprotein P are less flocculent than those of the type-specific fraction M and appear as a diffuse cloud; the use of absorbed serum is, however, necessary to distinguish between these two forms of precipitate.

TABLE II.

*Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain S43 (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.*

	Volume of extracts and concentration of $\text{P}^e$	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture					Sera prepared with glossy culture				
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11				
Purified solution of homologous type-specific substance M from S43	0.4 cc.	++	++	+++	++	++	+++	++	±	±	±	+	±	±	±	±
	0.1 cc.	++	++	+++	++	++	+++	++	±	±	±	+	±	±	±	±
	0.025 cc.	++	+	++	+	++	+	++	±	±	±	+	±	±	±	±
Purified solution of species-specific carbohydrate C	0.4 cc.	++	++	±	++	+	++	+	++	++	++	++	++	++	++	++
	0.1 cc.	++	++	+	++	++	++	++	++	++	++	++	++	++	++	++
	0.025 cc.	+	++	+	++	++	++	±	++	++	++	+	++	++	+	+
Purified solution of nucleoprotein P	1 in 1,000	+	+	+	+	+	+	±	+	+	+	+	+	+	±	±
	1 in 4,000	+	+	+	+	+	+	±	+	+	+	+	+	+	±	±
	1 in 16,000	-	±	±	±	±	±	-	-	-	-	-	-	-	-	-

\* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.



produced traces of type-specific antibody when used as an antigen for immunizing rabbits.

TABLE III a.

*Passive Protection of Mice against S43 Matt Virulent by Three Kinds of Sera, Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.*

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
0.00001 cc.	†72	S	S	See	S	S	See	S	S	S	†24	S
0.0001 cc.	†24	S	S	Table	S	S	Table	†77	†90	S	†21	†27
0.001 cc.	†21	S	S	III b	S	†21	III b	S	S	†22	†21	S
0.01 cc.	—	S	S		†22	†21		S	†21	†21	†21	†21
0.1 cc.	—	†21	†29		†24	†21		S	†21	†21	†21	†21

In all tables the following symbols are used.

S indicates that the mice survived for 8 days and were then discarded.

† indicates death of mice.

Numerals indicate number of hours between time of injection and finding mice dead.

— indicates that the test was not done.

The mice received 0.5 cc. of serum intraperitoneally and 24 hours later test doses of virulent culture were injected into the peritoneal cavity.

TABLE III b.

*Passive Protection of Mice against S60 Matt Virulent (Same Type as S43) by Three Kinds of Serum Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.*

Test doses of virulent culture	Control normal mice	Serum prepared with S43 matt virulent culture A3	Serum prepared with S43 matt attenuated culture A6	Serum prepared with S43 glossy culture A11
0.000001 cc.	†45	S	S	S
0.00001 cc.	S	†45	S	†69
0.0001 cc.	†45	S	S	†45
0.001 cc.	†21	S	S	†23
0.01 cc.	†21	S	S	†21
0.1 cc.	†21	†21	†21	S

*Passive Protection of Mice.*—Table III a shows the degree of passive immunity conferred on mice by these sera against a virulent culture

of the homologous strain and Table III *b* shows the protection against a virulent culture of S60, a strain identified by Dochez, Avery and Lancefield (2) as belonging to the same type as S43.<sup>4</sup>

TABLE IV *a*.

*Active Immunization of Mice against S43 Matt Virulent by Vaccines Prepared with S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.*

Test doses of virulent S43 culture	Control normal mice	Mice previously inoculated with S43 vaccines*			Controls Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with matt attenuated culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	†31	S	S	†24	†28	†71
0.00001 cc.	†28	S	†29	†23	†23	†27
0.0001 cc.	†29	S	S	†23	†23	†23
0.001 cc.	†23	†80	†23	†23	†23	†23
0.01 cc.	†23	†23	†23	†23	†23	†23
0.1 cc.	†23	S	†47	†23	—	†23

\* Vaccines = undiluted 16 hour broth cultures heated at 56°C. for 1 hour.

TABLE IV *b*.

*Showing that the Actively Immune Mice Surviving the Test Recorded in Table IV *a* Were Not Protected against a Matt Virulent Culture of a Heterologous Strain S23.*

Test doses of virulent S23 culture	Control normal mice	Mice protected with S43 matt vaccines which had survived test doses of S43 matt virulent
0.000001 cc.	S	†23
0.00001 cc.	†23	†23
0.0001 cc.	†144	†23
0.001 cc.	†23	†23
0.01 cc.	†23	†23
0.1 cc.	†8	†23

The sera prepared against the matt virulent and matt attenuated cultures afforded an equal amount of protection to mice and the

<sup>4</sup> Most of the protection tests recorded in this paper were only done once and no attempt has been made to correct obvious discrepancies due to the natural resistance of individual mice by repeating the tests.

anti-glossy sera also showed some protective power. The small degree of protection conferred on mice by the anti-glossy sera corresponds to the small quantity of type-specific antibody contained in the sera (see Table II) and this coincidence becomes significant when we observe, from the study of strain S23 that serum, prepared with completely degraded glossy cultures which are devoid of type-specific substance, does not contain any type-specific antibody and does not protect mice.

*Active Immunization of Mice.*—Table IV records the results obtained when mice, actively immunized with the matt virulent, matt attenuated and glossy cultures, were tested for immunity.

Tryptic digest broth cultures of the three forms of Strain S43 were incubated for 16 hours and then killed by heating at 60°C. for an hour. These heat-killed cultures, undiluted and suspended in the broth in which they had grown, were inoculated intraperitoneally into three series of mice. During the 1st week the mice received 0.1 cc. doses on 4 successive days, during the 2nd week four 0.2 cc. doses and during the 3rd week four 0.4 cc. doses. 10 days after the final inoculation the active immunity of the mice was tested by intraperitoneal injection of graduated doses of the matt virulent culture. The controls for this experiment were (1) a series of normal untreated mice, (2) a series of mice inoculated with corresponding doses of tryptic digest broth, (3) a series of mice treated with the matt virulent vaccine of a heterologous strain, S23.

The mice vaccinated with the matt cultures in both the virulent and attenuated forms showed evidence of protection; but mice vaccinated with the glossy form were not protected against infection with the matt virulent cocci.

The specificity of active immunization is also demonstrated by this experiment. Vaccination with a heterologous matt virulent Strain S23 did not afford any protection against Strain S43 and Table IV *b* shows that there was no cross-immunity when protected mice surviving from the first experiment and immune to S43 were tested 10 days later for immunity to S23.

### *B. Strain S23.*

This strain, isolated from a case of bronchopneumonia at the same time as Strain S43, had been used by Andrewes, Derick and Swift (3) as the test organism for protection experiments, and in the course

of their work its virulence had been increased by passage through a number of mice.

Virulence tests showed that 0.000001 cc. of culture killed mice regularly and occasionally 0.0000001 cc. and even 0.00000001 cc. proved a fatal dose.

The colonies had an undulating contour which made observations on the light-reflecting properties of their surfaces difficult and this peculiarity persisted when the strain was converted to the glossy state. Cultures of the virulent form usually contained many pseudo-glossy colonies and, although a large number of plate cultures were examined, it was only rarely that either matt or glossy colonies appeared in typical forms.

Owing to these peculiarities we relied entirely on precipitin tests, by which the quantity of type-specific substance in a culture can be assessed, to judge when this strain was completely degraded to the variant form. The matt virulent strain was cultivated in homologous high titer anti-M serum and after 55 transfers in 50 per cent serum, concentrated extracts of the culture did not precipitate the homologous anti-M serum; and a single mouse passage did not cause the reappearance of type-specific substance. This variant culture, which, measured by the absence of type-specific substance, was completely degraded, was nevertheless partially virulent, 0.01 cc. being the M.L.D. for mice.

It was difficult to obtain a matt attenuated culture as the peculiarities of the strain made selection of colonies impossible. After nine transfers in homologous immune serum, containing only a small amount of type-specific antibody, the virulence of the culture was reduced from 0.000001 cc. to 0.001 cc. although the quantity of type-specific substance, measured by precipitin reactions, remained unchanged. This culture probably represented the matt attenuated form of Strain S23 as it was only slightly more virulent than the glossy variant; but owing to its high virulence, compared with the matt attenuated cultures of other strains, it was not used in our experiments.

*Precipitin Tests.*—Two series of rabbits were immunized with the matt virulent and glossy forms of Strain S23; Table V shows the precipitin reactions of their sera. It will be seen that all the sera



prepared against the matt virulent culture were precipitated by an extract containing the homologous type-specific substance but the sera prepared against the glossy variant remained perfectly clear

TABLE VI.

*Absorption of Anti-Matt and Anti-Glossy Sera with the Homologous Matt and Glossy Cultures. Strain S23.*

	Volumes of extracts	Serum B6 prepared with matt virulent culture of Strain S23			Serum B9 prepared with glossy culture of Strain S23		
		Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy	Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy
Purified solution of homologous type-specific substance M from S23	0.4 cc.	++±	—	++±	—	—	—
	0.1 cc.	++±	—	++±	—	—	—
	0.025 cc.	+	—	+	—	—	—
Purified solution of species-specific carbohydrate C	0.4 cc.	—	—	—	—	—	—
	0.1 cc.	—	—	—	+±	—	—
	0.025 cc.	—	—	—	++	—	—

TABLE VII.

*Passive Protection of Mice against S23 Matt Virulent by Two Kinds of Serum Prepared against S23 Matt Virulent and S23 Glossy.*

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
0.000001 cc.	†67	—	—	S	—	S	†30	†21	†31	†23	†29
0.000001 cc.	†23	S	S	S	S	S	†21	†24	†25	†20	†29
0.00001 cc.	†23	†23	†23	S	†23	S	†21	†21	†26	†20	†23
0.0001 cc.	†23	S	S	S	†26	S	†21	†21	†23	†20	†23
0.001 cc.	—	†28	S	†96	S	S	†21	†21	†23	†20	†23
0.01 cc.	—	†21	†21	†27	†21	†72	†21	†21	†23	†20	†23
0.1 cc.	—	†21	†21	†21	†21	S	†21	†21	—	†20	†23

when mixed with the same extract. Both kinds of immune sera were precipitated by the non-type-specific fractions P and C, the anti-glossy sera being as usual more uniformly rich in antibody to the carbohydrate fraction C.

The anti-matt serum (B6) and the anti-glossy serum (B9) were chosen from the two groups of sera for absorption experiments. Both sera were absorbed with homologous matt virulent cocci and also with homologous glossy cocci. Table VI shows the result of this experiment.

The type-specific antibody was completely removed from the anti-matt serum by absorption with the homologous matt culture but it was unaffected by absorption with the homologous glossy culture.

The anti-glossy serum contained no type-specific antibody but its non-type-specific antibody was completely removed by absorption with both forms of the homologous strain.

TABLE VIII.

*Active Immunization of Mice against S23 Matt Virulent by Vaccines Prepared with S23 Matt Virulent and S23 Glossy.*

Test dose of virulent S23 culture	Control normal mice	Mice previously inoculated with S23 vaccines		Controls Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	S	S	†24	†41	†41
0.00001 cc.	†63	†41	†21	†41	†26
0.0001 cc.	†23	†41	†22	†22	†21
0.001 cc.	†21	†110	†21	†21	†41
0.01 cc.	†21	†17	†17	†17	†17
0.1 cc.	†17	†13	†17	†22	†17

*Passive Protection of Mice.*—The sera prepared against the two forms of Strain S23 were used for mouse protection experiments with the homologous matt virulent culture as the test organism.

All the sera prepared against the matt virulent form afforded some degree of protection to mice but the anti-glossy sera did not confer any protection.

*Active Immunization of Mice.*—Two series of mice were inoculated with vaccines prepared from the matt and glossy forms of Strain S23; the dosage and technique were the same as those used in a similar experiment, previously recorded, with Strain S43. Controls con-

sisted of a series of normal mice, a series of mice inoculated with a heterologous matt virulent vaccine and a series of mice inoculated with broth (Table VIII).

In this experiment vaccines prepared from both forms of Strain S23 failed to confer immunity on the mice.

### C. Strain C203.

This strain, from the collection of the Laboratories of the New York State Department of Health, was originally isolated from a

TABLE IX.

*Precipitin Tests on Two Kinds of Sera, Prepared against C203 Matt Virulent and C203 Glossy with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.*

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture			Sera prepared with glossy culture			
		C1	C2	C3	C4	C5	C6	C7
Purified solution of homologous type-specific substance M from C203	0.4 cc.	+++	++	++	±	±	-	±
	0.1 cc.	++	+±	+	-	-	±	-
	0.025 cc.	+	+	±	-	-	-	-
Purified solution of species-specific carbohydrate C	0.4 cc.	-	-	-	-	+	-	+±
	0.1 cc.	-	-	-	++	+++±	-	+++
	0.025 cc.	±	-	-	+++±	++	-	+++±
Purified solution of nucleoprotein P	1 in 1,000	+±	±	+	++	+++±	+	+++±
	1 in 4,000	+	±	±	+±	+	±	+±
	1 in 16,000	-	-	-	±	±	-	±

case of scarlet fever and had been kept in stock culture for a considerable time before our experiments commenced. It was found to be entirely composed of typical matt colonies; and virulence tests showed that the M.L.D. for mice was 0.0000001 cc. or 0.00000001 cc.

The glossy variant form of this strain was obtained by selection of colonies combined with cultivation in 50 per cent homologous anti-M serum of high titer. After 27 transfers the virulence for mice had fallen from 0.0000001 cc. to 0.1 cc. and the culture formed typical glossy colonies; but traces of type-specific substance could still be



detected in unconcentrated HCl extracts. Continued efforts to secure a completely degraded culture did not cause any further decrease in the quantity of type-specific substance which the organisms contained.

Attempts to reduce this strain to the matt attenuated form were unsuccessful as any reduction in virulence was always accompanied by a partial loss of type-specific substance and by the appearance of atypical matt colonies which tended to assume the characteristics of glossy colonies.

*Precipitin Tests.*—Rabbits were immunized with the matt virulent and glossy forms of this strain; Table IX gives the precipitin reactions of the immune sera.

In this instance a departure was made from the technique used for immunizing rabbits with the other three strains; the organisms were washed to remove the toxin as the use of unwashed broth cultures of highly toxic strains involved the loss of a relatively large proportion of rabbits during immunization.

The results of precipitin tests were similar to those previously recorded in reference to other strains; the sera prepared with the matt virulent organisms gave good precipitates with the homologous type-specific substance M but only traces of precipitate with the non-type-specific fractions C and P; on the other hand sera prepared with glossy organisms gave only traces of precipitate with the type-specific substance M and comparatively good precipitates with the non-type-specific substances C and P.

The traces of type-specific antibody in the anti-glossy sera were a natural sequel of failure to secure this strain in the completely degraded form.

*Passive Protection of Mice.*—Table Xa shows the results of protection tests with the different kinds of sera. All the anti-matt sera showed evidence of protective power and the anti-glossy sera with one exception also protected mice to some extent. This protection by sera prepared against the glossy form of Strain C203 is in harmony with the results of precipitin tests and with the original observation that the culture was not fully degraded but still contained type-specific substance. Table Xb shows the specificity of protection by the anti-glossy sera. On reference to Table Xa it will be seen that

TABLE X a.

*Passive Protection of Mice against C203 Matt Virulent by Two Kinds of Sera Prepared against C203 Matt Virulent and C203 Glossy.*

Test doses of virulent culture Strain C203	Control normal mice	Sera prepared with matt virulent culture			Sera prepared with glossy culture				Control mice inoculated with normal rabbit sera	
		C1	C2	C3	C4	C5	C6	C7	N1	N2
0.0000001 cc.	†24	S	S	S	†26	†25	S	S	S	†22
0.000001 cc.	†21	†24	S	†120	†22	S	S	S	†24	†28
0.00001 cc.	†21	S	S	S	†21	†25	S	†21	†21	†26
0.0001 cc.	†21	S	†168	S	†21	†25	†69	†21	†21	†21
0.001 cc.	†21	†24	†21	S	†21	S	†93	†21	†21	†21
0.01 cc.	—	†21	†21	†24	†21	†21	†21	†21	†21	†21

TABLE X b.

*Specificity of Passive Protection of Mice by Anti-Glossy (C203) Serum.*

Test doses of virulent culture Strain S23	Control normal mice	Serum prepared with glossy culture of Strain C203 C6
0.0000001 cc.	†53	†51
0.000001 cc.	†51	†22
0.00001 cc.	†22	†26
0.0001 cc.	†22	†56
0.001 cc.	†22	†22
0.01 cc.	—	†22

TABLE XI.

*Active Immunization of Mice against C203 Matt Virulent by Vaccines Prepared with C203 Matt Virulent and C203 Glossy.*

Test dose of virulent C203 culture	Control normal mice	Mice previously inoculated with C203 vaccines		Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared from matt virulent culture	Vaccine prepared from glossy culture	Vaccine prepared from heterologous matt virulent culture	Inoculated with broth
0.0000001 cc.	†25	S	—	†40	†40
0.000001 cc.	†21	S	†24	†20	†25
0.00001 cc.	†22	S	†64	†16	†18
0.0001 cc.	†16	S	†20	†21	†16
0.001 cc.	†16	S	†16	†16	†16
0.01 cc.	†16	S	†17	†16	†16
0.1 cc.	†16	†20	†16	†16	†16

Serum C6 was probably the best anti-glossy serum for protection against the homologous matt virulent form of Strain C203; a series of mice was, therefore, injected with this serum and their immunity to a heterologous matt virulent Strain S23 was subsequently tested. Table Xb shows that Serum C6 afforded no protection against the heterologous Strain S23.

*Active Immunization of Mice.*—Mice were vaccinated with the different forms of Strain C203 in the manner previously described; and their immunity was tested by inoculating graduated doses of a matt virulent culture of the same strain.

Table XI shows that vaccination with matt virulent organisms produced immunity against subsequent infection by the same strain; but vaccination with homologous glossy organisms, or with heterologous matt virulent organisms did not cause any immunity.

#### *D. Strain London.*

This strain, isolated by blood culture from a case of puerperal septicemia, had been kept in stock for about a year before our experiments began; and plate cultures showed that it was composed of a mixture of matt and glossy colonies. A pure matt culture, capable of killing mice in a dose of 0.01 cc., was obtained by selecting a suitable colony and the matt virulent form (M.L.D. 0.000001 cc.) was then prepared by passing this culture through 47 mice.

The matt attenuated culture, which was entirely composed of typical matt colonies indistinguishable from those of the virulent form and failed to kill mice when 0.5 cc. was injected intraperitoneally, was obtained by combining the selection of typical matt colonies with cultivation at 41°C.

The glossy variant was obtained by daily subcultivation of the original strain on agar slants without any selection of colonies or cultivation in immune serum. After 148 transfers on agar the culture was entirely composed of typical glossy colonies, and it was avirulent for mice (M.L.D. 1.0 cc.); but precipitin tests with concentrated HCl extracts of the organisms showed that this culture still retained traces of the type-specific substance M.

*Precipitin Tests.*—Table XII gives the precipitin reactions of the sera of three sets of rabbits immunized with the three forms of this

TABLE XII.  
*Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.*

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture								Sera prepared with glossy culture				
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17
Purified solution of homologous type-specific substance M	0.4 cc.	++	++	++	++	++	++	++	++	++	++	++	±	-	±	-	-	±
	0.1 cc.	++	+	++	++	++	+	+	++	±	+	+	-	-	-	-	-	-
	0.025 cc.	+	±	-	±	±	-	-	-	-	-	±	-	-	-	-	-	-
Purified solution of species-specific carbohydrate C	0.4 cc.	±	-	-	-	-	-	++	-	-	-	-	-	++	-	++	++	-
	0.1 cc.	++	-	-	-	+	-	++	-	-	±	±	±	++	++	++	++	±
	0.025 cc.	++	-	-	±	++	-	++	-	-	++	++	++	++	++	++	++	±
Purified solution of nucleoprotein P	1 in 1,000	±	+	±	+	+	+	+	±	±	+	+	+	+	+	+	+	+
	1 in 4,000	±	±	±	±	±	±	±	±	±	-	+	+	±	±	±	±	±
	1 in 16,000	+	±	-	±	±	-	±	-	-	-	-	-	-	±	-	-	-



strain; and it will be seen that, as in previous similar experiments, the anti-matt sera were relatively rich in antibody to the type-specific substance M; while the anti-glossy sera had a preponderance of antibody to the non-type-specific fraction C.

*Passive Protection of Mice.*—Table XIII gives the results of protection tests with the sera prepared against the different forms of Strain London: both kinds of anti-matt sera protected mice against infection with the homologous matt virulent organisms but the anti-glossy sera, although they contained traces of type-specific antibody had little protective power.

TABLE XIV.

*Active Immunization of Mice against the Matt Virulent Form of Strain London by Vaccines Prepared from the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).*

Test dose of matt virulent culture of Strain London	Control normal mice	Mice previously inoculated with vaccines prepared from Strain London in the following forms			Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Matt virulent	Matt attenuated	Glossy	Heterologous matt virulent	Broth
0.0000001 cc.	†41	—	†89	—	†41	†41
0.000001 cc.	†27	†89	†41	†41	†25	†41
0.00001 cc.	†22	†70	†22	†24	†41	†27
0.0001 cc.	†22	S	†22	†22	†24	†17
0.001 cc.	†24	†65	S	†22	†22	†17
0.01 cc.	†17	†22	†17	†22	†17	†17
0.1 cc.	†17	†17	†17	—	†17	—

*Active Immunization of Mice.*—Table XIV shows the results of vaccinating mice with the three forms of Strain London and with matt virulent organisms of a heterologous strain. In this instance a final dose of 0.2 cc. of heat-killed culture was substituted for 0.4 cc. as used in other experiments because the mice began to lose weight during immunization. In this experiment, although there was little evidence of immunization by any of the vaccines, the mice vaccinated with the matt virulent organisms survived longer than any of the controls.

## DISCUSSION.

Matt and glossy cultures of four strains of hemolytic streptococci, belonging to different serological types, were used to immunize rabbits. Only one of the four glossy cultures, Strain S23, was completely degraded to the point at which no trace of type-specific substance could be detected in highly concentrated HCl extracts of the organisms. The glossy cultures of the other three strains were not completely degraded as extracts from these organisms contained traces of the type-specific substance M.

Precipitin tests with the antisera of the four strains gave the following results: (1) All the anti-matt sera, whether prepared with virulent or attenuated organisms, contained antibody to the type-specific substance M. (2) The anti-glossy sera, prepared with cultures which were not fully degraded, were relatively deficient in type-specific antibody and gave either negative or weakly positive precipitin reactions with the type-specific substance M. (3) The five sera prepared with the completely degraded glossy form of Strain S23 did not contain any type-specific antibody. (4) The type-specific antibody was completely removed by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Experiments on passive immunization of mice showed that all the anti-matt sera had some protective power against infection with homologous matt virulent organisms. Some of the anti-glossy sera, prepared against strains which were not fully degraded, also protected mice but none of the anti-glossy sera prepared against the completely degraded glossy form of Strain S23 afforded any protection against infection with the homologous virulent organisms.

No exact parallel was established between the anti-M titer and the protective power of immune sera but high titer anti-M sera usually gave good protection.

It might be supposed that protection against infection with matt organisms by anti-matt sera and the absence of protection by anti-glossy sera merely represented specificity which could be demonstrated between different forms of the same strain and that anti-glossy sera would protect against infection with glossy organisms

better than anti-matt sera. Three of the strains used in this investigation were unsuitable for experiments to test this possibility because the glossy organisms were completely avirulent for mice. The completely degraded glossy variant of Strain S23 was, however, relatively virulent for mice (M.L.D. 0.01 cc.) and comparison was, therefore, made between the protective power of anti-matt and of anti-glossy sera against infection with the homologous glossy organisms. No evidence was obtained that either the anti-matt sera or the anti-glossy sera had any protective power against infection with glossy organisms.

There is considerable evidence that both passive protection and active immunity were antibacterial and not antitoxic; this evidence may be summarized as follows:

1. All the strains produced toxin to some extent but the protective action of the antisera was type-specific; sera prepared with toxigenic strains did not protect against infection with heterologous strains of equal toxigenicity. The immunity due to vaccination was also type-specific.

2. The toxigenicity of the matt form and of the glossy form of Strain S23 was determined quantitatively by intracutaneous tests in human subjects, and it was found that the matt and the glossy forms were approximately equal in their power to produce skin-reactive toxin. In spite of this equality, anti-glossy sera possessed no protective power against infection with homologous matt virulent organisms.

3. The anti-matt sera against Strain C203 were prepared with washed bacteria, and the production of antitoxin was, therefore, limited, but in spite of this limitation the sera protected against the highly toxigenic homologous strain but not against the weakly toxigenic heterologous Strain S23.

4. Active immunization of mice with whole broth cultures of the matt and of the glossy forms of the scarlet fever Strain C203 showed that, although the two forms were approximately equal in toxigenicity, the matt vaccine produced a high degree of immunity while the glossy vaccine produced no immunity against infection with homologous virulent organisms.

These observations, which show that the matt varieties of hemolytic



streptococci are type-specific while the glossy variant forms are not type-specific, are in agreement with the work of Andrewes. He also isolated two varieties of hemolytic streptococci corresponding to our matt and glossy forms, which, in a preliminary communication (4), he designates "rough" and "smooth" with the reservation "that they must not be supposed to correspond with the rough and smooth forms of *B. coli* and *Salmonella*." Using a special technique, he was able to establish by agglutination and absorption experiments that the "rough" forms exhibit considerable specificity; that the "smooth" forms of different strains are all alike serologically, and that the "rough" and "smooth" forms of a given strain are serologically distinct.

A comparison of his results with those recorded in this paper shows that his type-specific agglutination with hemolytic streptococci of the matt variety is in agreement with our observation that large quantities of the type-specific substance M are found in matt organisms; also his observation that anti-glossy serum agglutinates all strains of hemolytic streptococci, when in the glossy form, with complete impartiality is in agreement with our observation that anti-glossy sera contain more antibody to the non-type-specific fractions than anti-matt sera.

#### SUMMARY.

The matt and the glossy forms of four strains of hemolytic streptococci were used to immunize rabbits.

Precipitin tests showed that rabbit sera prepared against matt organisms, whether virulent or avirulent for mice, contained type-specific antibody while sera prepared against completely degraded glossy organisms contained no type-specific antibody.

Type-specific antibody was removed from the sera by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Passive protection experiments on mice showed that anti-matt sera were protective and anti-glossy sera non-protective against infection with homologous virulent organisms.

Vaccination of mice with matt organisms rendered them immune to subsequent infection with homologous virulent cultures; but vaccination with glossy organisms established no active immunity.

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## PHYSIOLOGICAL ONTOGENY.

### A. CHICKEN EMBRYOS.

#### XIV. THE HYDROGEN ION CONCENTRATION OF THE BLOOD OF CHICKEN EMBRYOS AS A FUNCTION OF TIME.

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These experiments continue to illustrate an interest in the processes of growth which are exhibited in earlier communications (1). We have turned now to the study of a more general function and have studied the changes which take place in the hydrogen ion concentration of the blood of chicken embryos. Chickens were selected in the expectation that the new data might form part of the information already accumulated and might serve the purpose of further inferences and wider generalization.

The ages of the embryos studied ranged between 8 and 20 days of incubation. It was impossible to secure sufficient volumes of blood before this age to permit measurements to be made, even when the vessels were successfully punctured by the needle of the syringe. We regret this omission from our data the more because we are aware of the fact that it is precisely at this early age, from 5 to 8 days, that information is desirable. It may be possible at another time to return to this period of the life cycle when methods other than those now available are at our disposal. During the period of hatching and in the first days of life, other difficulties beside that of drawing blood were encountered. Although no difficulty was experienced at the time of hatching in obtaining blood, it was found necessary to carry out this procedure either while the animals struggled or when they had been rendered quiet by the use of anesthetics such as amytal. In both cases, the blood must inevitably be unnatural as the result of dis-

turbance of the oxidation processes, due either to excess lactic acid formation during struggling, or in anesthesia to moderate asphyxia and to diminished rate of blood flow. We learned in the course of our experiments to avoid including measurements taken of the blood of embryos after they had been injured or when they were in a moribund state. In both cases there is a strong tendency for the blood to be more acid than is natural. We came in the end to recognize the presence of these states from examining the blood alone. It has occurred to us that the inclusion of such estimations might result in the publication of erroneous curves.

The incubation of the eggs of White Leghorn hens was arranged in the manner formerly described (2). The ages of the embryos were estimated with care, subject to the difficulties which are usually encountered even when eggs are brought promptly to the incubator after having been laid. The embryos were subsequently studied without removal from the incubator room. The glass electrodes (3), employed in measuring the hydrogen ion concentration, were also installed here. Embryos and their shed blood were, in short, constantly at  $38^{\circ} \pm 0.5^{\circ}\text{C}$ . The wires from the electrodes were led, appropriately sheathed, from the incubator room, through its wall to the adjoining room, where the electrometer was housed.

Blood was drawn either from a vein (arterial blood) or from an artery (venous blood) through a needle into the barrel of a syringe. Into the end of the syringe, a second one which contained the piston was fitted identical with the first. The syringe system from point of needle to piston was filled with paraffin oil. A second person manipulated the piston.

In opening the eggs it was necessary to avoid the occurrence of hemorrhage. Help toward this end was obtained by painting the shell membrane, after enough of the shell was chipped away, with paraffin oil. By this means two objects were attained; first, it was possible to locate blood vessels, and second, capillary hemorrhage was prevented. It is not certain how the effect in preventing hemorrhage took place nor whether the method was free from danger; capillaries may have closed because the membranes, coated with oil, became impermeable to the passage of gases. Usually the amount of the surface of the egg affected in this way was small. When, through

clearing the membrane, blood vessels became visible, it was often possible to insert a needle into them without further dissection—obviously a great advantage. Both oxygenated and reduced blood was obtained from embryos of all ages; in older embryos, both varieties were often drawn from the same animal.

Measurements of the reaction of the blood could be undertaken with volumes no greater than 0.25 cc. When withdrawn the blood

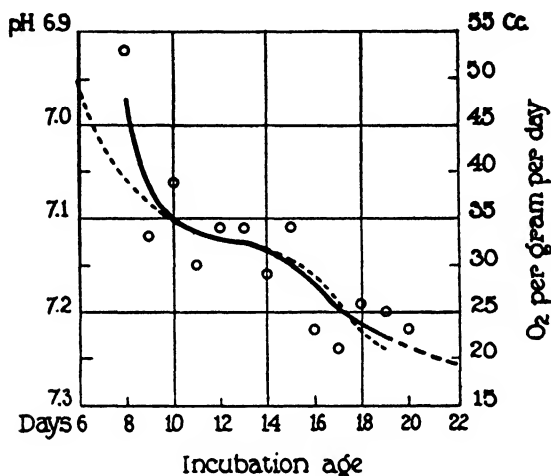


FIG. 1. A curve is shown (the solid line) indicating the course of the change in hydrogen ion concentration in the arterial (reduced) blood of chicken embryos. The circles represent the average pH of the blood at each day of incubation. In the middle of the curve, corresponding to the period between 10 and 14 days, there is a flattening of the curve. A curve (dotted line) published by Murray showing alterations with time of oxygen consumption, flattens at the same period; the ends of the two curves follow similar courses.

was plunged into Ringer solution already under oil in such proportions that the dilution of blood to solution was as 1 to 3 or 4. A small amount of sodium fluoride was likewise present in the test-tube to inhibit glycolysis. The effect of dilution to this extent in the value of the measurement, since it was not sufficiently known, has been studied. Preparations were made of undiluted and of diluted blood and estimated. The difference in value between the two was found

to be no more than a few hundredths of a pH. So diluted and prepared by vigorous stirring to prevent coagulation, the blood was transferred to the cup of the glass electrode. Measurements required so little time that the rate at which they could be made depended on the dissection of the embryos and the withdrawal of blood rather than on the manipulation of the apparatus.

We found a consistent change in the hydrogen ion concentration of the blood throughout that period of embryonic life which we examined. The general trend of the change was from acid to alkaline (Fig. 1 and Table I). At 8 days the pH averaged 6.92 and at 20 days 7.22. The lowest figure which was encountered was 6.75 in an 8 day embryo. We may point out that the lowest pH encountered so far in human beings is 6.95\* and was found in a patient in a moribund state (4). It should be pointed out that the value in the venous blood of an adult animal, when the blood was withdrawn from an animal lying quite still without the use of anesthesia was 7.30. It is important to notice the low value in embryos of younger age. It suggests inferences that may be drawn from investigations published by Warburg and his collaborators (5) in connection with measurements of rapidly growing tissues in general, in the case either of tumors or of embryonic tissues in the period when their mass is undergoing great increase. We may recall that in this connection Warburg's measurements dealt with the rate of glycolysis measured in terms of the rate of lactic acid formation. There is to be found in this process not only increase in acid formation but actually an increase in the acid reaction. We are not in position to say that these two phenomena are necessarily mutually dependent. We shall return to this point.

We have for comparison with the results in fowl, studied the blood both arterial and venous, of the fetuses of cats. We were unable, unfortunately, to secure a continuous series so that data could be systematically collected according to time. The fetuses that were studied differed in age but the dating was too uncertain to allow us to draw a curve of the rate of change which takes place. The measurements which were made covered the range from pH 7.04 to 7.31. These it will be perceived lie within that found in chicken embryos. It

\* In a personal communication Dr. D. D. Van Slyke tells us that this is the lowest pH of which he is aware.

appears that the reaction which is found in chicken embryos is in no way peculiar to this family, but is characteristic of embryonic and perhaps of rapid growth in general. Okuneff (6) has in point of fact shown that plant tissue in the stage of regeneration after injury likewise exhibits a low pH.

TABLE I.

*The Changes in Hydrogen Ion Concentration of the Blood in Chicken Embryos.\**

Incubation age	Reduced blood		Oxygenated blood	
	Average pH	No. of observations	Average pH	No. of observations
<i>days</i>				
8	6.92	13	7.03	8
9	7.12	4	7.18	5
10	7.06	6	7.23	3
11	7.15	4	7.19	1
12	7.11	4		
14	7.16	2	7.19	1
15	7.11	5		
16	7.22	4	7.22	3
17	7.24	3		
18	7.19	5	7.23	4
19	7.20	4	7.20	2
20	7.22	5	7.23	2
Adult	7.30		7.34	

\* The standard deviations from the averages are not given. They are wider than would be encountered in the normal resting human subject. At the 8th day for instance, the range is 6.75 to 7.11. We have not given the figures in detail because in our opinion they are in a sense misleading. We believe the wide range is due to the impossibility not only of dating accurately the age of the embryos but also of excluding the possibility of injury. There seems now no reason to doubt that the pH of embryos, if they could be known to be of the same age, would be similar within a narrow range.

On our curve of change in the hydrogen ion concentration, we have inserted the points indicative of the change in oxygen consumption obtained by Murray (7) as expressive of change in the rate of metabolism. It is difficult to escape the impression that the two observe, while not a parallel, at all events a somewhat similar course. As a result of this comparison arises the suggestion either that both are

expressions of the same underlying process, or that they stand in some causal relation to each other. That oxygen consumption and lactic acid formation are both expressions of the metabolic function, is already known. The fact that the curve expressing the change in hydrogen ion concentration takes the form it does in respect to Murray's curve of oxygen utilization raises the question of the possibility of the existence of a relation between lactic acid production and the concentration of hydrogen ions. We are not in possession of measurements which place the formation of lactic acid in a determining position in respect to reaction but have been led to think of this possibility on account of the facts which have just been described.

#### SUMMARY AND CONCLUSION.

We have studied by means of glass electrodes the hydrogen ion concentration of the blood of chicken embryos from 8 to 20 days. When plotted as a curve, the average data show that a constant change takes place in the measurement, being acid at the beginning of this period and becoming alkaline toward its end. The acid reaction we think was characteristic not only of the embryos of fowl, but of the fetuses of cats and indeed as Warburg has shown of rapidly growing tissue in general. We have suggested the possibility, though we have no data to substantiate the suggestion, that the hydrogen ion concentration may under conditions like these, seeing that the curve of change resembles that of oxygen consumption, be expressive of changes in the rate of metabolism.

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## STUDIES OF UREA EXCRETION.

### II. RELATIONSHIP BETWEEN URINE VOLUME AND THE RATE OF UREA EXCRETION BY NORMAL ADULTS.<sup>1</sup>

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#### DEFINITION AND CALCULATION OF THE MAXIMUM AND STANDARD BLOOD UREA CLEARANCES

Investigations by Marshall and Davis (1914), Pepper and Austin (1915), Addis and Watanabe (1916), and by Addis, Barnett, and Shevky (1918) have shown that when the urine volume is fairly large the rate of urea excretion is directly proportional to the blood urea content. Expressed in other words, with abundant urine the urea excretion per minute equals the urea contained in a constant volume of blood. This volume of blood in a normal adult is about 75 cc.

Austin, Stillman, and Van Slyke (1921) demonstrated in three normal subjects that the direct ratio between blood urea content and urea excretion rate holds only when the urine volume is above a certain limit (about 2 cc. per minute in adults), which they called the "augmentation limit." When the urine volume fell below the augmentation limits of the subjects studied, the urea excretion rate was found to fall also, and on the average, in proportion to the square root of the volume: e.g., if, blood urea remaining constant, the urine volume were diminished from 2 to 0.5 cc. per minute, the urea excretion rate would be halved.

The conception of these authors is confirmed in this paper by observation on 7 other subjects, and the augmentation limit in normal adults has been found to range from 1.7 to 2.5 cc. of urine per minute.

When the urine volume output is at any point *above the augmenta-*

<sup>1</sup> The first paper of this series was by Austin, Stillman, and Van Slyke (1921) on "Factors Governing the Excretion Rate of Urea."

*tion limit*, urea excretion proceeds at maximum speed, and the output per minute represents the urea content of a maximum blood volume. This blood volume, averaging in normal men about 75 cc. per minute, we shall for convenience term the *maximum blood urea clearance*, or *simply the maximum clearance*. It represents the volume of blood which one minute's excretion suffices to clear of urea when the urine volume is large enough to permit a maximum urea output. The value of the maximum clearance,  $C_m$ , is calculated from the observed urea concentrations of the blood and urine,  $B$  and  $U$ , and the urine volume,  $V$ , in cubic centimeters per minute, by the formula,

$$\text{Maximum clearance} = C_m = \frac{U V}{B}$$

The concentration ratio,  $\frac{U}{B}$ , indicates the number of cubic centimeters of blood the urea content of which is represented in 1 cc. of urine.  $\frac{U}{B} \times V$  therefore indicates the number of cubic centimeters of blood represented in the urea content of the  $V$  cubic centimeters of urine excreted in 1 minute.

*Below the augmentation limit* the volume of blood, the urea content of which is represented in one minute's excretion, (the blood urea clearance per minute) is not a constant, but varies, on the average, in proportion to the square root of the urine volume. In order to compare excretions below the augmentation limit, therefore, they must either be observed with a standard, constant, urine volume output, or, if observed with other urine volumes, the excretion rates must be corrected for the urine volume effect. It is practically impossible to fix the urine volume at a definite standard, but, by means of the square root rule of Austin, Stillman, and Van Slyke, the urea excretion that would accompany such a standard urine volume can be calculated from the excretion measured with any other volume below the augmentation limit.

The formula for the calculation is developed as follows:

If  $C$  is the observed blood urea clearance (the cubic centimeters of blood, the urea content of which is excreted in 1 minute) with any

urine volume output,  $V$ , below the augmentation limit, then with the standard urine volume,  $V_s$ , the corresponding *standard clearance*,  $C_s$ , may be calculated by the square root rule as

$$C_s : C = \sqrt{V_s} : \sqrt{V}$$

or

$$C_s = C \sqrt{\frac{V_s}{V}}$$

The standard urine volume that we have adopted is 1 cc. per minute. This value not only simplifies calculation because it is represented by unity: it presents itself also as a natural standard, because it is approximately the average rate of urine excretion for normal adults (1440 cc. for 24 hours; Addis (1923) found 1345 cc. as the average). Consequently the observed volumes will on the average differ less from it than from any other volume. Substituting therefore 1 for  $V_s$ , we obtain

$$C_s = C \sqrt{\frac{1}{V}}$$

For convenience in calculation it is desirable to substitute in place of the observed clearance,  $C$ , the figures directly determined by analysis, viz. the urea concentrations,  $U$  and  $B$ , in urine and blood respectively, and the urine volume,  $V$ . As shown above in connection with the calculation of the maximum clearance, the observed clearance is estimated as  $C = \frac{UV}{B}$ . We therefore substitute  $\frac{UV}{B}$  for  $C$  in the equation  $C_s = C \sqrt{\frac{1}{V}}$  and obtain:

$$\text{Standard clearance} = C_s = \frac{U}{B} \sqrt{V}$$

*For the condition that the urine volume is below the augmentation limit.*

*The standard clearance indicates the efficiency with which the kidneys excrete urea when the urine volume is at the average normal level of 1 cc. per minute. The maximum clearance indicates the maximum efficiency*

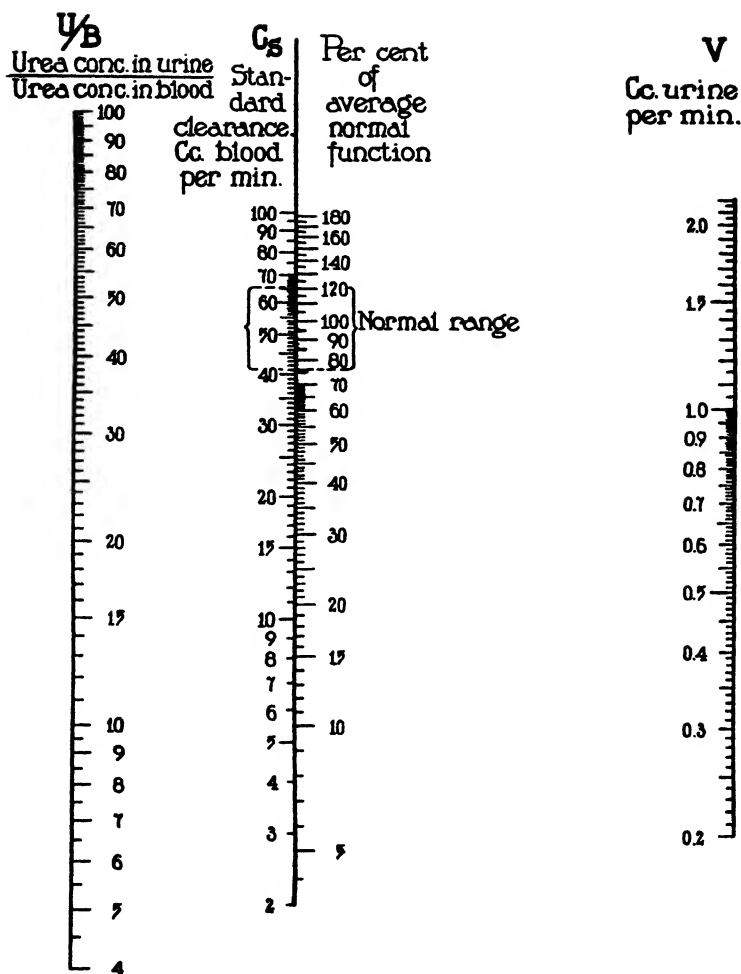


FIG. 1. LINE CHART FOR CALCULATING MAXIMUM BLOOD UREA CLEARANCE,

$$C_m = \frac{U V}{B}, \text{ FROM } U, B, \text{ AND VALUES OF } V \text{ ABOVE THE}$$

AUGMENTATION LIMIT

Connect observed  $U/B$  and  $V$  values by a straight line. Where the line cuts the inner scale read  $C_m$  value or per cent of average normal renal function.

For subjects differing markedly from usual adult size, a correction is introduced by multiplying the observed  $V$  value by the factor  $\frac{1.73}{\text{sq. m. surface area}}$  (see next

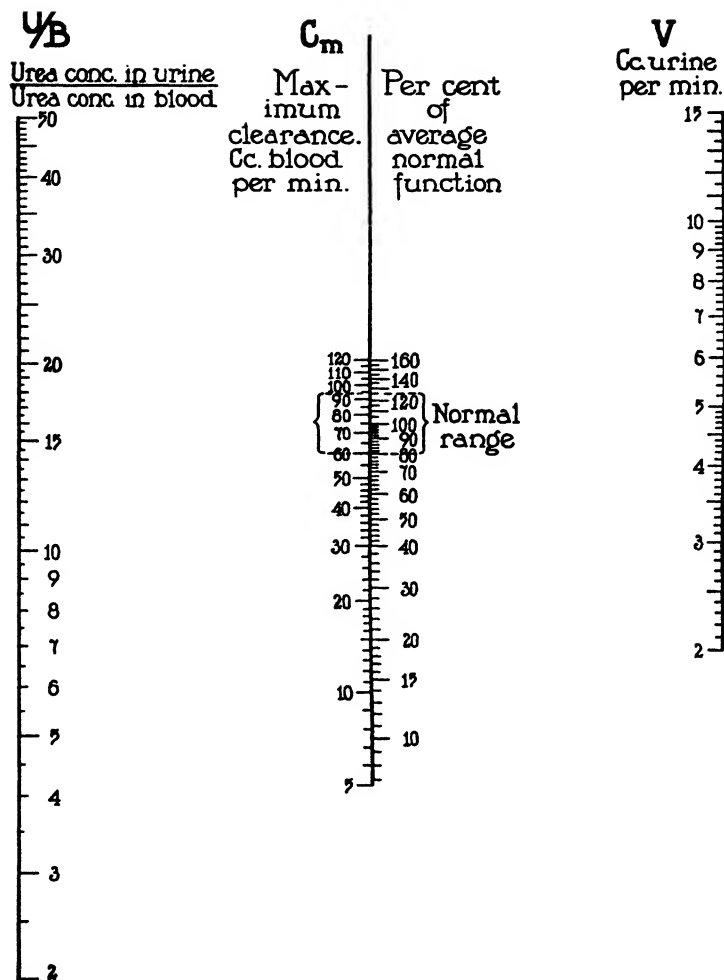


FIG. 2. LINE CHART FOR CALCULATING STANDARD BLOOD UREA CLEARANCE,

$$C_s = \frac{U \sqrt{V}}{B}, \text{ FROM } U, B, \text{ AND VALUES OF } V \text{ BELOW THE}$$

AUGMENTATION LIMIT

Connect observed  $U/B$  and  $V$  values by a straight line. Where the line cuts the inner scale read  $C_s$  value or per cent of average normal renal function.

For subjects differing markedly from usual adult size, a correction is introduced by multiplying the observed  $V$  by the factor  $\frac{1.73}{\text{square meters surface area}}$  (see next paper), and using the  $V$  value thus corrected for the calculation of  $C_s$ .

of urea excretion with high urine volumes. The maximum clearance is normally about 40 per cent greater than the standard clearance, the mean values being 75 cc. of blood per minute for the maximum and 54 cc. for the standard. Usually, though not always, in pathological conditions both values are affected to approximately the same degree.

For use in the above formulae for calculating  $C_s$  and  $C_m$ , any convenient units of urea or urea N concentration, e.g. grams per liter, milligram per 100 cc., may be used to express the urea concentrations,  $U$  and  $B$  so long as the *same* unit is used for both  $U$  and  $B$ . This follows from the fact that in each formula  $U$  and  $B$  appear only in the ratio  $\frac{U}{B}$ , so that both  $U$  and  $B$  may be multiplied by any factor without changing the value of  $\frac{U}{B}$  ratio, or of the  $C_s$  or  $C_m$  calculated therefrom.

The unit for expressing values of  $V$ , however, can not be changed without changing the numerical values of  $C_s$  and  $C_m$ .

#### CALCULATION OF CLEARANCE VALUES

If the urine volume exceeds 2 cc. per minute, as observed in an adult, or as corrected for body size (see next paper) in a child, the *maximum clearance* is calculated.

If the volume thus observed or corrected is less than 2 cc. per minute, the *standard clearance* is calculated.

It is advantageous as a rule to calculate both clearances in percentages of the mean normal  $C_s$  and  $C_m$ . Urea excretions observed with ordinary urine volumes and calculated in terms of  $C_s$  are thus rendered directly comparable with excretions observed with large urine volumes and hence calculated in terms of  $C_m$ . Furthermore the percentage values thus calculated express directly percentages of average normal renal efficiency.

The percentage of average normal  $C_m$  is obtained by dividing the absolute  $C_m$  value by the mean normal  $C_m$ , 75, and multiplying by 100. Similarly the percentage of average normal  $C_s$  is obtained by dividing the absolute  $C_s$  by 54 and multiplying by 100.

$$\text{Per cent of average normal } C_m = \frac{100 UV}{75B} = 1.33 \frac{UV}{B}$$

$$\text{Per cent of average normal } C_s = \frac{100 U \sqrt{V}}{54B} = 1.85 \frac{U \sqrt{V}}{B}$$

*Graphic calculation of  $C_m$  and  $C_s$  by charts of figure 1 and figure 2.* Both the absolute and percentage  $C_m$  and  $C_s$  values are most readily calculated graphically by means of a slide rule, or by means of the line charts in figure 1 and figure 2 respectively. When the charts are used it is necessary to calculate by arithmetic only the value of the quotient  $\frac{U}{B}$ , and of  $V$  in cubic centimeters per minute, corrected for body size as outlined in the next paper if the subject is a child. A thread stretched taut across figure 1 or figure 2 intersecting the observed values of  $\frac{U}{B}$  and  $V$  on the outer scales then crosses the inner scale at a point indicating both the absolute clearance and the percentage of normal.

*Arithmetical calculation of  $C_m$  and  $C_s$ .* For arithmetical calculation of the standard clearance the following values of the square root of  $V$  are given covering the range below the augmentation limit.

$V$ cc. per minute	$\sqrt{V}$	$V$ cc. per minute	$\sqrt{V}$
0.2	0.45	1.2	1.10
0.3	0.55	1.3	1.14
0.4	0.63	1.4	1.18
0.5	0.71	1.5	1.23
0.6	0.78	1.6	1.27
0.7	0.84	1.7	1.30
0.8	0.89	1.8	1.34
0.9	0.95	1.9	1.38
1.0	1.00	2.0	1.42
1.1	1.05	2.1	1.45

*Example of calculation of a normal maximum clearance*

$$\begin{aligned} \text{Blood urea N} &= 15.6 \text{ mgm. per 100 cc.} = B \\ \text{Urine urea N} &= 321.0 \text{ mgm. per 100 cc.} = U \\ \text{Urine volume} &= 210 \text{ cc. per hour} \\ &= 3.5 \text{ cc. per minute} = V \end{aligned}$$

$$C_m = \frac{UV}{B} = \frac{321 \times 3.5}{15.6} = 72 \text{ cc. of blood cleared of urea per minute}$$

$$\text{Per cent of average normal function} = 1.33 \times 72 = 96 \text{ per cent}$$

*Example of calculation of a normal standard clearance*

$$\text{Blood urea N} = 14.7 \text{ mgm. per 100 cc.} = B$$

$$\text{Urine urea N} = 750 \text{ mgm. per 100 cc.} = U$$

$$\text{Urine volume} = 50 \text{ cc. per hour}$$

$$= 0.83 \text{ cc. per minute} = V$$

$$C_s = \frac{U\sqrt{V}}{B} = \frac{750 \times 0.91}{14.7} = 46 \text{ cc. of blood cleared of urea per minute}$$

$$\text{Per cent of average normal function} = 1.85 \times 46 = 85 \text{ per cent}$$

*Technique for determining the blood urea clearance as a measure of renal efficiency.* The necessary data are the concentrations of urea in blood and urine, and the volume of urine excreted in a measured time. The manner in which these 3 values are secured may be varied to suit conditions. As a routine procedure, however, we have found the following satisfactory:

The subject is not subjected to any previous routine, except that vigorous exercise is avoided and the previous meal should be a moderate one, preferably without coffee, which Addis and Drury (1923) have found may increase the blood urea clearance. The most desirable time of day, when excretion is least liable to fluctuations, is found according to MacKay (1928) in the hours between breakfast and lunch. The patient remains quiet while the urine is collected during two succeeding periods of 1 hour each. The chief source of error is probably the possibility of incomplete emptying of the bladder, either at the beginning or end of a period. The collection of two urine specimens affords a check on this factor. A few minutes before the end of the first hour a blood sample is drawn. Its urea content is used for calculation of the clearances during both periods. This usage is permissible, because under the conditions of the test the blood urea does not change greatly during an hour.

The maximum clearance is calculated if the urine volume observed in an adult, or if the corrected volume  $V \times \frac{1.73}{\text{Sq. m. surface area}}$



in a child, exceeds 2 cc. per minute. (See accompanying Paper III of this series.)

The standard clearance is calculated if the urine volume, corrected in the case of a child, is less than 2 cc. per minute.

*Physiological and pathological significance of maximum and standard blood urea clearances.* The excretion rate observed with the average normal maximum blood urea clearance is what would be obtained if 75 cc. of blood per minute passed the kidneys, and all its urea were excreted. Actually a larger volume of blood perfuses the kidneys, and only a certain fraction of its urea is removed. Picard (1856) found that in dogs the urea content of blood from the renal vein was half that of blood from the artery. If a similar relation held for man, the average normal renal blood flow could be estimated at 150 cc. per minute, 50 per cent of the urea of this blood being excreted with large urine volumes and 35 per cent with the average urine volume of 1 cc. per minute.

*Decrease in the volume of blood cleared of urea per minute in pathological conditions must be due to one of two causes: either the volume of blood per minute passing through the kidneys is diminished, or the proportion of its urea removed during the passage is less than normal.* In cardiac decompensation presumably the flow factor is responsible for lowered renal function; and in glomerular nephritis the damaged renal vessels make diminished flow again seem certain. Whether a decrease in the proportion of urea removed from the blood also occurs or not, we have not at present the basis to surmise.

#### THE VARIABILITY OF THE BLOOD UREA CLEARANCE, AND ITS SIGNIFICANCE

The fact that in a given individual the probable variation of the standard blood urea clearance is  $\pm 10$  per cent, and that the maximum variation is much greater, indicates, as pointed out by Austin, Stillman, and Van Slyke (1921), that other factors in addition to blood urea concentration and urine volume affect urea excretion. Some of these factors were studied by Addis and Drury (1923), who found that the maximum clearance was increased by ingestion of a mixed meal, milk, caffeine, or glutamic acid, and decreased by pituitrin or

very large doses of adrenalin. The effect of adrenalin, however, was shown in rabbits by Addis, Barnett, and Shevky (1918) to vary with the dosage; up to a certain maximum it increased urea output, but greater amounts depressed the output. Ordinarily Addis (1917) believed that adrenalin and pituitrin act as antagonists in regulating renal activity.

Such influences may vary the blood clearance per minute in either of two ways. They may vary the renal blood flow without altering the percentage of blood urea removed at each passage through the kidneys. Or they may so influence the activity of renal cells that variations do result in the percentage of blood urea removed at each passage. The questions, whether and how the percentage of urea removed from the blood in the kidneys can be influenced, awaits experimental proof.

It is evident that the urea excretion rate is influenced by other factors in addition to blood urea content and urine volume, and that an erroneous impression would be created by the clearance formulae if they were assumed to express with mathematical exactness the complete effects of all factors influencing urea excretion. The width of the range of normal variation indicates the contrary. The formulae are only expressions of the effects of two factors, blood urea content and urine volume, which are in continual action and appear to be ordinarily of chief importance in regulating the urea output.

To minimize variations due to other factors Addis (1922) in determining the maximum clearance gives water and urea to the fasting subject at the beginning of a 6 hour period, and analyzes specimens of blood and urine collected during the last 3 hours of the period, during which diuresis is maintained by water drinking. In determining the standard clearance in this laboratory we have thus far set no conditions, except that the subject should be at rest and should have avoided coffee and other obvious diuretics during the preceding hours of the day. The limits of variation in our results, reported below, apply to these conditions. It appears possible that by standardizing conditions more completely the range of variation could be narrowed.

## THE HISTORICAL DEVELOPMENT OF UREA EXCRETION TESTS OF RENAL FUNCTION IN NEPHRITIS

That the relatively simple conceptions of the maximum and standard blood urea clearances outlined in the preceding pages are logical developments of a long course of observation, experiment, and study by many investigators is indicated by the following historical summary.

In 1827 Bright first described the disease which has been called after him. The year after, 1828, Wöhler performed the synthesis of urea. This was the first time any organic substance had been synthesized *in vitro*.

The coincidence of these two scientific events at once created an interest in urea excretion in Bright's disease. Christison (1829) found the serum urea content increased in 3 patients out of 6, and further observed, that in these 3 cases the rate of urea excretion was slower than in the 3 others. Bright (1836) found a serum urea concentration of as much as 1500 mgm. per cent in a case of uremia. He noted that the concentrations of urea in the serum and in the urine of this patient were the same, and that while the former was increased, the latter was only about one-third of the ordinary normal value. These findings were soon confirmed by other authors, and in 1851 Frerichs, in his monograph, stated that while in acute cases of Bright's disease urine urea concentration and urea output were both normal, in chronic cases both were lower than in normal subjects on the same diet. In such cases he believed that the determination of the urea concentration of the urine could be of diagnostic value. A few years later diagnostic use of the blood urea concentration was recommended by Picard (1856).

During the second half of the nineteenth century the investigation of Bright's disease was chiefly concerned with the morphological and circulatory changes. The few isolated comments upon the usefulness of the determination of urine urea concentration (Green, 1885) or excretion rate (Cruise, 1890; Guyon, 1892) were only exceptions to the general rule.

In about 1900 interest turned to the functional aspect of disease, and the study of urea excretion was resumed. The point of view, however, was now different. Determination of the blood urea content and the urea excretion rate were carried out (separately or combined) not only for purposes of qualitative diagnosis, but in order to furnish quantitative information about the degree of functional impairment of the kidneys. The unrelated measurement of excretion rate or of urea concentration in urine was soon found to be unsatisfactory for this purpose. The reason was that both these factors are too dependent on the rate of protein catabolism and the urine volume. The influence of protein catabolism was fully recognized at the time, but that of urine volume was not.

Achard and Paiseau (1904) brought patients into nitrogen equilibrium and then added to the diet 20 grams of urea daily for some days. They measured the urea

output per 24 hours, and noted the rapidity with which the superimposed amount of nitrogen was excreted. The procedure was, however, laborious, the results not very consistent. The method was abandoned, to be revived occasionally by later authors.

Other authors turned their attention to the blood urea, and neglected the excretion. The determination of blood urea was introduced into clinical medicine by Strauss (1902) and by Widai and Javal (1904). For diagnostic purposes determination of blood urea concentration has an advantage over determination of urine concentration, in that with ordinary urine volumes the blood figure is less dependent on fluctuations of water output. Other factors being constant, however, the blood urea content is proportional to the rate of protein catabolism. If a given subject breaks down into urea half as much protein daily his average blood urea will be half as high, given a constant urine volume. If the urine volume increases within the ordinary range (below the augmentation limit), the blood urea will be further diminished, increased water output washing out more urea from the blood. Both of these factors are likely to be operative in nephritis to prevent a rise in blood urea proportional to renal destruction. MacKay and MacKay (1927) in fact report data (which our own confirm) showing that many nephritics do not show blood ureas definitely above the normal maximum until more than 60 per cent of renal function has been lost.

The conception of comparing simultaneous urea determinations in blood and urine was introduced by Gréhan (1904) who used the concentration ratio  $\frac{U}{B}$  as an expression of renal functional ability. However, the immense effects of urine volume changes on the urea concentration,  $U$ , in urine were not considered, in consequence of which even approximate constancy can not be obtained with this ratio. The use of the  $\frac{U}{B}$  ratio was revived by Harrison (1922), who emphasized that the most consistent results were gained when the urine volumes were below 150 to 100 cc. per hour. This restriction reduces the inconsistencies introduced into the  $\frac{U}{B}$  ratio by urine volume changes, but also limits the conditions under which observations can be made.

Ambard and Weill (1912) were the first to include both urea output and urine volume in attempting quantitatively to relate urea excretion to blood content. They found that urea excretion in normal subjects, and also in nephritics, was governed by two laws, relating output to blood and urine concentrations respectively. These laws were combined into the urea excretion formula of Ambard and Weill (1912), which, with numerical constants omitted, is:

$$K = \frac{B}{\sqrt{D} \sqrt{U}}$$

$B$  and  $U$  represent, as above, concentration of urea in urine and blood, and  $D$  indicates rate of urea excretion (*débit*). Since  $D = U \times V$ , Ambard's formula can be written

$$K = \frac{B}{U^{\frac{1}{3}} V^{\frac{2}{3}}}$$

For the sake of comparison with our standard clearance formula we take the reciprocal, and have

$$\frac{1}{K} = \frac{U^{\frac{1}{3}}}{B} \sqrt{V}$$

In this form the similarity can readily be seen of Ambard's formula with our present formula,  $C_s = \frac{U}{B} \sqrt{V}$ , for calculating standard blood clearance. Although the influence of urine volume was not directly investigated by the French authors, a correction for urine volume is nevertheless contained in their formula and is brought out through its transcription as above. The volume factor  $\sqrt{V}$  is valid, however, only for ordinary urine volumes of less than 2 cc. per minute. Above this point the volume factor should be  $V$ , and for higher urine volumes the Ambard formula becomes increasingly more inaccurate. The use of  $U$  in the  $\frac{1}{3}$ th power instead of in the first appears, in the light of present results, to be a complication diminishing rather than enhancing the accuracy of the formula. The introduction of the Ambard formula was, however, an important advance in estimating the effect of urine volume on urea excretion.

F. C. McLean (1915) put the Ambard formula into a more convenient form by squaring and inverting it, and adding a numerical constant, which made 100 the average normal value of this "urea secretory index."

Addis and Watanabe (1916) found a wide range of variation in results calculated from normal subjects by Ambard and Weill's formula, and presented data indicating the degree of inaccuracy of the two basic assumptions. Addis believed the urea excretion ratio,  $\frac{D}{B} = \frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. blood}}$ , to be independent of urine volume (1917), and recommended its determination under certain standard conditions (1922) as a test of kidney function. These standard conditions will, except in rare pathological conditions, keep the urine volume well above the augmentation limit, and cause  $\frac{D}{B}$  to represent the maximum blood clearance.

The existence of the augmentation limit was first recognized by Austin, Stillman, and Van Slyke (1921) who showed, first, that Addis' urea excretion ratio is independent of urine volume only when this is above the augmentation limit of about 2 cc. per minute, and, second, that with urine volumes below this point the excretion rate varies most nearly in proportion to the square root of the volume.

In the present paper we confirm these results on normal subjects, and in an accompanying paper we show that they hold true for nephritics also.

H. MacLean and de Wesselow (1919, 1920) in the interest of simplicity reverted to a single determination, the urea concentration in the urine, as a test of renal function. These authors prescribed certain standard conditions for its determination, designed to make the values more consistent. They gave 15 grams of urea with 100 cc. of water, and noted whether or not the urine urea concentration in the 2 subsequent hours rose above 2 per cent. If it did, they considered the kidneys fairly efficient. Gross errors due to dilute urines were excluded by rejecting tests in which the second hour's urine volume exceeded 150 cc. Their procedure was admirably adapted to its primary purpose, the rapid examination of large numbers of soldiers. In the study of nephritic patients, however, the method invites error by neglect of the blood urea. For example, if urinary function is so low that only a tenth the normal blood volume is cleared of urea per hour, the urea output will nevertheless be normal if the blood urea concentration is ten-fold the ordinary. Hence the urinary concentration will also be normal, if the volume is not increased. For this reason, in the terminal stages of nephritis, with high blood urea content, a urinary urea concentration within ordinary normal ranges may be observed, despite tremendously reduced renal ability.<sup>2</sup> The interpretation of figures for urea concentration in urine is therefore uncertain, unless the blood urea content is known, as well as the urine volume.

The historical sequence in which the different urea determinations were introduced as indicators of renal function, and the conditions under which they were best applicable, are summarized in table 1.

*Numerical relation of the present standard clearance to previously used forms of the Austin-Stillman-Van Slyke formula.* The formula  $\frac{U}{B} \sqrt{V}$

expresses the number of cubic centimeters of blood of which the urea content is concentrated into 1 cc. of urine, when urine excretion is at the average normal rate of 1 cc. per minute. The mean normal numerical value of 54 indicates that under these conditions the kidneys concentrate the blood urea 54-fold. The standard clearance thus may be interpreted as a measure of the concentrating power, as well as the excreting ability, of the kidney. For this reason the value now called the standard clearance has, in a number of papers from this laboratory (e.g. Hiller, McIntosh, and Van Slyke (1927)) been called the "*concentration index*." The term "standard clearance" is at pres-

<sup>2</sup> "Selbst die prozentige Ausscheidung des N kann noch relativ gut erscheinen, ja 1 per cent betragen, wenn bereit die tödliche Vergiftung begonnen hat" (p. 166 of Volhard and Fahr, 1914).

TABLE 1

*Determination used to indicate state of kidneys' ability to excrete or concentrate urea*

Functional test	Value determined	Variation, direct or inverse, with renal function	Conditions of applicability	Authors
Urea concentration in urine	$U$	Direct	Conditions assuring maximum concentration, viz., minimum urine volume, sufficient blood urea	Frerichs (1851) Guyon (1892) McLean and de Wesselow (1919)
Urea concentration in blood	$B$	Inverse	Normal diuresis and ordinary rate of protein catabolism	Picard (1856) Strauss (1902) Widal (1904)
Concentration ratio between urine and blood	$\frac{U}{B}$	Direct	Minimum or standard urine volume	Gréhant (1904) Harrison (1922)
Ambard's urea-secreatory constant. Original form.	$\frac{B}{\sqrt{D \times \frac{70}{\text{weight}} \times \sqrt{\frac{U}{25}}}}$	Inverse	Below augmentation limit	Ambard and Weill (1912)
Addis' urea excretion ratio. (Maximum blood clearance)	$\frac{D}{B}$ or $\frac{UV}{B}$	Direct	Large urine volumes, above augmentation limit of about 2 cc. per minute	Addis and Watanabe (1916)
Standard blood clearance	$\frac{U}{B} \sqrt{V}$	Direct	Moderate and small urine volumes, below augmentation limit of about 2 cc. per minute	Austin, Stillman and Van Slyke (1921)

\*  $U$  and  $B$  in this formula are expressed in grams of urea per liter urine and blood respectively. Freed of arbitrary constants, reduced to terms of  $U$ ,  $B$ , and  $V$ , and inverted for comparison with other formulae, Ambard's formula becomes  $\frac{U^{\frac{1}{2}}}{B} \sqrt{V}$ .

ent preferred, partly because when used in conjunction with "maximum clearance" it suggests more clearly the difference in conditions under which the two respective urea excretion rates are determined.

In the above papers the formula used in calculating the "index" was  $\frac{U}{B} \sqrt{\frac{V}{W}}$  instead of  $\frac{U}{B} \sqrt{V}$ . However, in the  $\frac{V}{W}$  ratio used the volume unit was cubic centimeters per hour per kilogram, which, for a person of 60 kgm. weight, is the same as cubic centimeters per minute. Hence the values of  $\frac{U}{B} \sqrt{\frac{V}{W}}$  in the above papers are approximately interchangeable with those of the present  $C_s = \frac{U}{B} \sqrt{V}$ . They deviate therefrom in proportion as  $\sqrt{W}$  deviates from  $\sqrt{60}$ , but the fact that unusually low or high body weights influence the value  $\frac{U}{B} \sqrt{\frac{V}{W}}$  only in proportion to their square roots, and not their first powers, diminishes the effect on the calculated clearance. E.g., a person of 50 kgm. would weigh 17 per cent less than one of 60; but the effect of this weight difference on the value of the index  $\frac{U}{B} \sqrt{\frac{V}{W}}$  is only 9 per cent. Our present practice, discussed in the next paper, is to correct for wide divergence from average size by multiplying  $V$  by the factor  $\frac{1.73}{\text{Sq. m. surface area}}$ .

The standard clearance  $\frac{U}{B} \sqrt{V}$  is, except for omission of the weight correction, identical with the excretion constant  $\frac{D}{B\sqrt{VW}}$  of Austin, Stillman and Van Slyke. If in  $\frac{D}{B\sqrt{VW}}$  the factor  $D$  is replaced by its equivalent,  $UV$ , the formula changes to  $\frac{U}{B} \sqrt{\frac{V}{W}}$ . Omission of the weight correction,  $W$ , simplifies it to  $\frac{U}{B} \sqrt{V}$ . The original numerical values of the excretion constant  $\frac{D}{B\sqrt{VW}}$ , or  $\frac{U}{B} \sqrt{\frac{V}{W}}$ , of these authors differed from the present clearance, and from the above discussed concentration index, because a different urine volume unit,  $\frac{V}{W} =$  liters per 24 hours per kilogram, was used. A given excretion rate expressed in cubic centimeters per minute is represented by a figure  $\frac{1000 W}{1440}$ , or  $0.694 W$ , times as large as that ex-



pressing the same rate in terms of liters per 24 hours per kilogram. The average weight of the 4 men studied by Austin, Stillman, and Van Slyke was 72 kgm. If we substitute this for  $W$  in the factor  $0.694 W$ , we find that a given excretion expressed in the terms of the above authors is to be multiplied by exactly 50 to correct it into cubic centimeters per minute. In order to convert the average value of the excretion constant obtained by Austin, Stillman, and Van Slyke into terms of the present standard clearance, the former value is consequently to be multiplied by  $\sqrt{50}$ , or 7.13. Their average normal constant of 7.5 therefore corresponds to a standard clearance of 53.6 cc. of blood per minute, which is nearly identical with the value calculated from the additional data yielded by other normal adults in this paper.

*Numerical relationship of the present maximum clearance to the excretion ratio of Addis.* The excretion ratio of Addis,

$$\frac{\text{urea in 1 hour's urine}}{\text{urea in 100 cc. blood}},$$

indicates  $\frac{1}{100}$  the number of cubic centimeters of blood, the urea content of which is represented by 1 hour's secretion. The excretion ratio is determined by Addis under conditions of large urine volumes, so that the value represents maximum clearance. A normal hourly excretion equivalent to the urea content of 5000 cc. of blood corresponds to an Addis ratio of 50, which was found by him (1922) to be the average normal value. Our maximum clearance, expresses  $\frac{1}{60}$  the cubic centimeters of blood, of which the urea content is represented by 1 hour's excretion. The Addis ratio is therefore converted into maximum clearance values, of cubic centimeters of blood cleared of urea per minute, by multiplying the Addis ratio by  $\frac{100}{60}$  or 1.67. The mean normal value of his ratio, found by Addis to be about 50 in young men, corresponds therefore to a per minute maximum blood clearance of about  $1.67 \times 50 = 83$  cc., which does not differ greatly from the average of 75 cc. found in our normal subjects.

#### THE CORRELATION BETWEEN URINE VOLUME AND UREA EXCRETION

The influence on urea output of urine volume changes below the augmentation limit, observed by Austin, Stillman, and Van Slyke, has

been questioned by Addis and his collaborators. Addis and Drury (1923) studied the relationship between  $V$  and the excretion ratio (or blood urea clearance). They found that in rabbits changes in volume down to 2 cc. per hour had no influence on the observed clear-

ance,  $\frac{UV}{B}$ . Of the 3 human subjects studied, however, only one was

observed with urine volumes below 120 cc. per hour, which is the usual augmentation limit according to our data. In this subject they found the blood clearance somewhat lower with urine volumes below 50 cc. per hour than with volumes above 64 cc. per hour. Since no regular quantitative relationship between excretion and urine volume was demonstrable from their data, however, these authors concluded that the increase in urea excretion with increase in urine volume, observed over the lower volume ranges by themselves and by Austin, Stillman, and Van Slyke, was due merely to the fact that certain factors stimulated both water and urea excretion: the excretion rates of these two substances Addis and Drury conceived to be independent of each other.

As opinions still are divided we have considered it desirable to increase the number of observations covering the influence of urine volume on urea excretion in normal subjects. We have attempted to limit the factors influencing excretion as nearly as practicable to one, water. In some of the experiments data were also obtained on the effect of urea ingestion, which, however, was not observed to affect significantly the clearance values obtained.

#### EXPERIMENTAL

We have examined 5 normal persons between 20 and 30 years of age, all in good health and without any history of kidney disease. We also have reexamined another normal subject, now 44 years of age, (Van Slyke), on whom data were first published by Austin, Stillman, and Van Slyke, six years ago.

During each experiment (except those on Van Slyke) the person examined was kept in bed. The reasons for this were, first, that changes of position are said to influence the water excretion through the kidneys (White, Rosen, Fischer and Wood (1926)) and, second, that the kidney function of patients is nearly always examined while they are in bed, so it seems more correct to compare them with normals studied under similar conditions.

In some of the experiments urea was taken between 6 and 8 a.m.

The test began at 9 a.m. and went on for from one to seven hours. During this time the subject voided at the end of each hour, and a sample of blood (usually 0.2 cc. from the ear lobe) was drawn 10 minutes before the middle of each 1-hour period. The intention was to obtain a blood urea figure representing as nearly as possible the average value during the hour when the urine was formed. The urine takes a certain time to flow from the renal tubules to the bladder. The error which is due to this factor is lessened when the blood is taken somewhat before the middle of the collection period.

At about 8 a.m. the subjects were allowed to take bread, butter and jam. At noon bread, butter, jam, fruit and vegetables were given. Addis and Drury (1923) found that ingestion of milk or coffee accelerated urea excretion appreciably; consequently we have avoided giving them during an experiment. Addis and Drury found moderate sugar ingestion to be without effect. We have not observed any significant effect of the above, chiefly carbohydrate, meals on the urea excretion rate in our subjects.

The variations in urine volume were obtained by controlling the water intake. Each patient was examined on 2 or more days. When minimal urine volumes were desired no fluids were given from the previous evening till noon or later. High volumes were obtained by giving water freely hour by hour, either from early morning or in the afternoon following a period of desiccation. No other factors were varied, except on occasions when urea was given. As will be seen, the urea was without discernible influence on the clearance values. The variations obtained in the blood clearances with varying urine volumes may, we believe, be attributed to the variations in water regime.

Our attention has been particularly directed to the possibility of such changes in blood clearance occurring, during sudden increases or decreases of water output, as were found in dogs under certain conditions by Bourquin and Laughton (1925). These authors observed exceedingly high clearances during the onset of diuresis and a period of depressed clearances when diuresis subsided and during more or less of the postdiuretic period. It will be seen from table 1, that although sudden changes in urine volume occurred in several of our experiments, no changes in clearance of the kind described by Bourquin and Laughton are found in our data on human subjects.

The concentrations of urea in the urine and in most of the blood samples were estimated by the gasometric urease method of Van Slyke (1927). Most of the blood analyses were performed on 0.200 cc. samples by the micro-technique. In experiments numbers A 7 to A 11, however, the blood was drawn by venous puncture, and the

urea concentration was estimated on samples of 3 cc. with the aeration urease method of Van Slyke and Cullen (1914).

#### RESULTS AND DISCUSSION

The conditions and results of all our experiments are given in tables 1 to 4.

The results for each of the 6 subjects investigated by us, and for one other from the literature (Rehberg, 1926), have been plotted in figures 3 to 9 with clearance values as ordinates and  $\sqrt{V}$  as abscissae. In order to simplify the plotting by obtaining straight line curves we have laid off as abscissae values of the square root of the urine volume. According to the square root rule, this procedure should enable one to express the relationship between urine volume and blood clearance as a rising straight line below the augmentation limit; and it will be seen in the graphs that such is the case. Above the augmentation limit volume has no effect, and the excretion curve becomes a horizontal line.

The curves have been drawn in the following manner. The mean value of the clearance  $\frac{UV}{B}$ , in cubic centimeters of blood containing the amount of urea excreted per minute, for all points above the augmentation limit is taken, and at the corresponding height above the horizontal axis, and parallel to it, a line is drawn. Then for all points to the left of the augmentation limit the standard clearance  $\frac{U}{B} \sqrt{V}$  is calculated, and the mean value is taken. This average determines the height of the curve at  $V = 1$  cc. Through the corresponding point on the vertical line representing  $V = 1$  cc., and through the zero point, a straight line is drawn. The position of the augmentation limit is calculated as the intersection point between this slanting line and the horizontal line first drawn.

In this way we have calculated augmentation limits from the data given by Austin, Stillman, and Van Slyke on Austin and Van Slyke our own data on six normal subjects, and finally the data given by Rehberg (1926) on himself, that were collected by him for quite other reasons, but can be used for our purpose as well.

TABLE 2  
*Data of experiments*

Experiments	Time	$V$ Urine volume	$U$ Urine urea nitrogen	$B$ Blood urea nitrogen	$\frac{UV}{B}$ Ob- served clear- ance*	$C_s = \frac{U\sqrt{V}}{B}$ Standard clearance, calculated for $V = 1$ , from observed clearances below aug- mentation limits	Per cent of average normal clearance, taken as $C_s = 54$ , $C_m = 75$
		cc per minute	mg per 100 cc	mg per 100 cc	cc blood per minute	cc blood per minute	per cent
Experiment Number A 7 L. L. Ca. 8:15, breakfast with ca. 100 cc. of water	9-10	0 82	1003	13 1	64 3	69.3	128
Experiment Number A 8 L. L. 6 a.m., 15 grams urea and 500 cc of water. 7, 8, 9, 10 and 11 a.m., 500 cc. of water each time	9-10	12 33	238	31 7	92 7*		124*
	10-11	10 33	202	29 2	71 5*		95*
	11-12	9 75	209	28 0	72 9*		97*
Experiment Number A 9 L. L. Ca 8:15, breakfast with ca. 100 cc. of water	9-10	0 57	1460	17 6	47 0	62 6	115
Experiment Number A 10 L. L. Ca. 8:15, breakfast with ca. 100 cc. of water	9-10	0 47	1260	20 5	28 7	42.2	78
Experiment Number A 11 L. L. Ca 8:15, breakfast with ca. 100 cc. of water	9-10	0 58	?	?	43 2	56.7	105
Experiment Number 2 L. L. 7 a.m., 15 grams urea. 11 a.m., 20 grams urea and 200 cc. of water	9-10	1 33	1231	26 3	62 4	54 0	100
	10-11	1 83	1063	25 5	76 5*	56 4	104
	11-12	3 58	857	40 3	76 0		101*
	12-1	3 62	852	38 5	80 0*		107*
	1-2	2 03	1115	34 1	66 5*		89*

\* Clearance figures marked \* represent maximum clearance values, determined when  $V$  was above the augmentation limit for the subject. For augmentation limits see table 4.

TABLE 2—Continued

Experiments	Time	V Urine volume	U Urea nitrogen	B Blood urea nitrogen	$\frac{UV}{B}$ Ob- served clear- ance*	$C_s = \frac{U\sqrt{V}}{B}$ Standard clearance, calculated for $V = 1$ , from observed clearances below aug- mentation limits	Per cent of average normal clearance, taken as $C_s = 54$ , $C_m = 75$
		cc. per minute	mg. per 100 cc.	mg. per 100 cc.	cc. blood per minute	cc. blood per minute	per cent
Experiment Number 1	9-10	7.17	415	39.3	75.5*		101*
J. F. M.	10-11	8.08	324	38.5	68.0*		91*
7 a.m., 30 grams urea and	11-12	4.00	842	46.6	72.3*		96*
500 cc. of water. 10 and	12-1	2.42	1321	44.9	71.2*		95*
11 a.m., 12 noon and 1	1-2	1.63	1398	36.7	62.2	48.6	90
p.m., 5 grams, urea each	2-3	1.67	1273	42.5	49.8	38.7	72
time							
Experiment Number 8	9-10	0.75	1568	25.1	46.8	54.1	100
J. F. M.	10-11	0.92	1564	29.0	49.5	51.8	96
7:15 a.m., 15 grams urea.	11-12	1.08	1260	29.9	45.7	43.8	81
12:45 p.m., lunch. 1 and	12-1	0.60	1185	24.6	29.0	37.3	69
2 p.m., 1000 cc. of water	1-2	1.03	1366	24.7	57.2	56.2	104
each time	2-3	8.58	193	21.9	75.7*		101*
Experiment Number 3	9-10	1.67	1068	25.8	69.2	53.5	99
A. H.	10-11	1.42	1034	25.5	57.5	48.3	89
7 a.m., 15 grams urea. 11	11-12	3.67	593	32.3	67.3*		90*
a.m., 20 grams urea and	12-1	7.87	264	40.0	52.2*		70*
200 cc. of water. 1:30	1-2	2.08	1095	34.2	67.0	46.2	85
p.m., lunch and 200 cc. of	2-3	2.42	901	31.7	68.8*		92*
water							
Experiment Number 25	9-10	0.57	679	13.5	28.5	37.9	70
A. H.	10-11	0.57	747	13.2	32.0	42.7	79
8:30, breakfast. 12 noon,	11-12	0.63	800	12.6	40.2	50.4	93
lunch and 1000 cc. of	12-1	1.63	495	13.3	60.8	47.5	88
water. 1 p.m., 500 cc. of	1-2	10.83	87.3	13.6	77.5*		103*
water	2-3	9.07	88.8	13.1	61.5*		82*
Experiment Number 5	10-11	0.60	1074	15.6	41.3	53.3	99
W. N.							
7 a.m., breakfast and 15							
grams urea in 75 cc. of							
water. Could not void							
on time							

TABLE 2—Continued

Experiments	Time	$V$ Urine volume	$U$ Urine urea nitrogen	$B$ Blood urea nitrogen	$\frac{UV}{B}$ Ob- served clear- ance*	$C_s = \frac{U\sqrt{V}}{B}$ Standard clearance, calculated for $V = 1$ , from observed clearances below aug- mentation limits	Per cent of average normal clearance, taken as $C_s = 54$ , $C_m = 73$
		cc per minute	mg per 100 cc	mg per 100 cc	cc blood per minute	cc blood per minute	per cent
Experiment Number 12	9-10	1 55	940	21 9	66 5	53 4	99
W. N.	10-11	1 13	864	32 5	30 2	28 3	52
7:15 a.m., 15 grams urea and 50 cc. of water. 8 a.m., breakfast and 100 cc of water							
Experiment Number 26	10-11	7 07	142	14 1	70 2*		94*
W. N.	11-12	8 67	90	14 6	53 5*		71*
8 a.m. breakfast and 1000 cc of water. 10 a.m., 500 cc. of water. 12 noon, lunch and 1000 cc of water	12-1	3 73	272	15 8	64 3		86*
	1-2	12 50	85 3	15 0	70 8*		95*
	2-3	8 70	93	14 0	57 6*		77*
Experiment Number 32	9-10	0 73	1010	16 0	46 4	53 9	100
W. N.	10-11	0 37	1070	15 4	25 5	42 3	78
8 a.m., breakfast. 12 noon, lunch with 200 cc. of water. 1:30 p.m., 250 cc. of water	11-12	0 77	974	14 7	50 8	58 2	108
	12-1	1 12	936	15 0	69 7	66 0	122
	1-2	2 80	390	14 9	73 2*		98*
	2-3	1 70	601	14 5	70 5*		94*
Experiment Number 9	10-11	2 42	725	30 5	57 3	37 0	69
J. C. B.	11-12	2 03	834	29 6	57 5	43 8	81
7 a.m., 15 grams urea in 50 cc. of water. 8 a.m., breakfast. 1 p.m., lunch and 250 cc. of water. 1:45 p.m., 250 cc. of water	12-1	1 23	916	22 6	50 0	45 0	83
	1-2	3 10	516	22 0	72 6*		97*
Experiment Number 15	9-10	1 80	808	24 2	60 2	44 8	83
J. C. B.	10-11	1 27	746	24 9	38 0	33 8	63
7 a.m., breakfast. 7:30 a.m., 15 grams urea and 50 cc. of water. 12:05 p.m., lunch and 1000 cc. of water. 1:05 p.m., 500 cc of water	11-12	1 75	775	24 6	55 3	41 8	78
	12-2	8 73	Lost	20 7			
	2-3	12 47	109	20 1	67 6*		90*
	3-4	3 75	302	17 3	65 3*		87*

TABLE 2—Continued

Experiments	Time	V Urine volume	U Urine urea nitrogen	B Blood urea nitrogen	$\frac{UV}{B}$ Ob- served clear- ance*	$C_s = \frac{U\sqrt{V}}{B}$ Standard clearance, calculated for $V = 1$ , from observed clearances below aug- mentation limits	Per cent of average normal clearance, taken as $C_s = 54$ , $C_m = 75$
		cc. per minute	mg. per 100 cc.	mg. per 100 cc.	cc. blood per minute	cc. per blood minute	per cent
Experiment Number 28	9-10	0.97	781	19.4	38.8	39.6	73
J. C. B.	10-11	1.00	731	19.2	38.1	38.1	71
7:30 a.m., breakfast. 12	11-12	1.25	656	18.9	43.3	38.8	72
noon, lunch	12-1	1.02	642	16.6	39.3	39.1	72
	1-2	0.80	746	17.2	34.7	38.8	72
Experiment Number 31	9-10	7.33	119.4	14.7	59.6*		79*
J. C. B.	10-11	7.58	103.3	12.5	62.6*		83*
7:30 a.m., breakfast and 500	11-12	8.75	83.4	12.3	59.3*		79*
cc. of water. 8:40, 10, 11	12-1	6.67	113.7	11.3	67.2*		90*
a.m., and 12 noon, 500	1-2	12.33	54.2	11.2	59.6*		79*
cc. of water each time. 12	2-3	11.67	52.2	10.5	58.0*		77*
noon, lunch. 1 p.m., 300							
cc. and 2 p.m., 200 cc. of							
water							
Experiment Number 33	10-11	0.80	765	13.7	44.6	49.8	92
D. V. S., 1927	11-12	1.33	640	11.6	73.6	63.6	118
8:30, breakfast. 12:45	12-1	1.07	619	12.7	52.0	50.3	93
p.m., lunch. No fluids.	1-2	0.73	775	11.2	50.7	59.2	110
Cutaneous blood	2-3	0.50	906	10.3	44.0	62.2	115
	3-4	0.70	806	9.4	60.0	71.7	133
	4-5	0.60	808	(9.4)	51.6	66.5	123
Experiment Number 34	9-10	6.41	153	13.1	74.8*		100*
D. V. S. 1927	10-11	16.25	58.6	12.3	77.4*		103*
8:30 a.m., breakfast with	11-12	13.25	86.0	12.3	92.6*		123*
800 cc. of water. 8:50	12-1	6.37	137	10.3	84.8*		113*
and 9:15 a.m. 200 cc.,	1-2	5.26	173	9.6	94.8*		126*
9:20 400 cc., 9:35 10 cc.,	2-3	1.32	438		60.2	52.4	97
and 10:40 200 cc., 10:45	3-4	2.77	281	(9.0)	86.4*		115*
400 cc. of water. 12:30							
p.m., lunch with 200 cc.							
of water. Venous blood							



TABLE 3  
Standard blood clearances in 9 other subjects

Initials	Height	Weight	Body surface	V Urine volume	U Urine urea nitrogen	B Blood urea nitrogen	Standard clearance $\frac{U\sqrt{V}}{B}$	Per cent of average normal clearance, taken as C <sub>s</sub> = 54
	cm	kgm	square meter	cc per minute	mgm per 100 cc	mgm per 100 cc	cc blood per minute	per cent
F C	166 3	67 1	1 75	0 32	1,671	25 2	37 3	69
				0 55	872	12 7	51 0	95
				0 83	822	15 9	46 4	86
				0 93	642	9 4	65 8	122
				1 03	947	16 8	52 1	96
Average							50 5	93
E V	160	58 1	1 60	0 25	1,282	20 2	31 7	59
				0 73	1,114	22 1	43 1	80
				1 57	605	14 8	51 0	95
Average							42 0	78
C A	178	64 4	1 80	0 48	939	9 8	66 5	123
				0 53	1,253	14 0	65 4	121
				0 63	854	10 7	63 1	117
Average							65 0	121
G S	173 5	61 2	1 72	0 46	1,433	18 8	51 6	96
				0 60	1,208	12 9	72 6	134
Average							61 1	113
J S	168 9	61 2	1 70	0 48	1,104	16 0	48 0	89
				1 03	777	16 9	46 7	86
Average							47 4	88
H C	173 8	58 9	1 70	0 52	1,414	15 1	67 2	124
				0 79	1,078	17 8	53 8	100
Average							60 5	112
C D	175 2	72 1	1 87	0 62	973	12 6	60 6	112
				0 67	1,205	21 0	46 8	87
Average							53 7	99
J P.	181	82 3	2 03	0 67	946	13 0	59 3	110
				0 70	1,132	13 5	69 5	129
Average							64 4	119
W. G.	177 7	61 7	1 78	0 29	1,432	16 3	47 5	88
				0 69	876	11 7	62 2	115
Average							54 9	102

TABLE 4

Summary of augmentation limit and standard clearance in normal adults

Subject	Body size			Augmentation limit Urine per minute	Standard clearance $C_s = \frac{U\sqrt{V}}{B}$ (from points below augmentation limit)					Per cent of average normal clearance, taken as $C_s = 54$ , $C_m = 75$
	Weight	Height	Surface area		Number of observations	Mean $C_s$ of individual	Probable deviation of a deviation of termination from mean $C_s$ of the individual	Mean $C_s$ corrected for body area		
	kgm.	cm.	square meters	cc.		cc. blood per minute	cc. blood per minute	per cent of mean $C_s$	cc. blood per minute	per cent
Austin.....	66	179.5	1.83	2.40	14	49.8	$\pm 2.92$	$\pm 5.86$	48.6	90
Van Slyke (1921)*....	72	174	1.86	(3.97)	14	(54.1)	$\pm 3.91$	$\pm 7.23$	(52.1)	96
Van Slyke (1927).....	72	174	1.86	2.05	8	59.5	$\pm 5.08$	$\pm 8.54$	57.3	106
J. F. M.....	60.3	164	1.65	2.35	7	47.2	$\pm 4.45$	$\pm 9.42$	48.3	89
L. L.....	58.3	171	1.68	1.98	6	56.9	$\pm 6.12$	$\pm 10.75$	57.1	106
W. M.....	72.2	182	1.43	1.67	7	50.8	$\pm 8.20$	$\pm 16.15$	48.2	89
J. C. B.....	57.3	171	1.67	2.55	11	40.1	$\pm 2.34$	$\pm 5.84$	40.9	76
A. H.....	52.7	159.5	1.53	1.98	7	46.6	$\pm 3.49$	$\pm 7.49$	49.5	92
Rehberg.....	80.0		1.95†	2.45	11	68.3	$\pm 5.11$	$\pm 7.48$	(64.3)†	119
McLean.....	70.0	175	1.85		14	62.4	$\pm 5.13$	$\pm 9.20$	(60.4)	112
F. C.....	67.1	166	1.75		5	50.5	$\pm 6.75$	$\pm 13.30$	50.3	93
E. V.....	58.1	160	1.68		3	42.0			42.6	79
C. A.....	64.4	178	1.72		3	65.0			65.3	121
G. S.....	61.2	173.5	1.72		2	61.1			61.4	114
J. S.....	61.2	169	1.70		2	47.4			47.9	89
H. C.....	58.9	174	1.70		2	60.5			61.1	113
C. D.....	72.1	175	1.87		2	53.7			51.8	96
J. P.....	82.3	181	2.03		2	64.4			59.4	110
W. G.....	61.7	177.7	1.78		2	54.9			54.1	100
Mean.....	65.2					54.5	$\pm 4.9$	$\pm 9.2$	53.8	100
Maximum.....	82.3					68.3	$\pm 8.2$	$\pm 13.3$	65.3	121
Minimum.....	52.7					40.1	$\pm 2.3$	$\pm 5.8$	40.9	76
Probable deviation of an individual mean from the mean of the group.....						$\pm 5.7$			$\pm 5.0$	$\pm 9.2$
Maximum observed deviation of an individual mean from mean of the group.....						+13.8			+11.5	+21.3
						-14.4			-12.9	-23.9

\* Excluded from average. See text.

† Calculated for an estimated height of 175 mm.

TABLE 5  
*Summary of data on maximum clearance in normal adults*

Reference to source of data	Subject	Maximum clearance $C_m = \frac{UV}{B}$					
		Number of observations	Mean $C_m$ of individual	Probable deviation of a determination from mean $C_m$ of individual		Mean $C_m$ corrected for surface area	Surface area
			cc blood per minute	cc blood per minute	per cent of mean $C_m$	cc blood per minute	square meters
(6)	Austin	6	77.2	$\pm 3.89$	$\pm 5.04$	73.2	1.83
Present paper	Van Slyke (1927)	6	85.1	$\pm 5.39$	$\pm 8.48$	79.2	1.86
	J F M	5	72.5	$\pm 2.17$	$\pm 2.99$	76.2	1.65
	L L	6	76.6	$\pm 6.14$	$\pm 8.02$	79.1	1.68
	W N	7	65.7	$\pm 6.32$	$\pm 9.62$	59.1	1.93
	J C B	9	63.6	$\pm 5.10$	$\pm 7.76$	66.1	1.67
	A H	5	65.5	$\pm 3.34$	$\pm 5.26$	74.0	1.53
Rehberg (15)	Rehberg	4	103.8	$\pm 9.92$	$\pm 9.55$	94.7	(1.90)
Mean of above data			76.3	$\pm 5.3^*$	$\pm 7.0$	75.2	1.753
Maximum			103.8	$\pm 9.9$	$\pm 9.6$	94.7	1.93
Minimum			65.5	$\pm 2.2$	$\pm 5.0$	59.1	1.53
Probable deviation of an individual mean from mean of group			$\pm 9.0$			$\pm 6.5$	
Addis (2)	Dru	33	93.0	$\pm 2.98$	$\pm 3.20$		
	Col	4	74.4	$\pm 2.94$	$\pm 3.95$		
	Sci	6	86.3	$\pm 4.97$	$\pm 7.76$		
	Add	21	76.5	$\pm 3.66$	$\pm 4.79$		
	Jen	4	83.4	$\pm 3.71$	$\pm 4.45$		
	Nor	4	97.3	$\pm 5.44$	$\pm 5.59$		
	Ges	4	68.2	$\pm 2.52$	$\pm 3.70$		
	Jac	4	99.1	$\pm 3.44$	$\pm 3.47$		
	New	7	64.4	$\pm 1.51$	$\pm 2.34$		
	Pis	6	83.7	$\pm 7.02$	$\pm 8.39$		
	Kol	7	96.5	$\pm 2.94$	$\pm 3.05$		
	Nye	6	73.1	$\pm 4.65$	$\pm 6.36$		
	Nak	4	76.3	$\pm 2.49$	$\pm 3.26$		
Mean of Addis data			82.3	$\pm 3.7$	$\pm 4.5$		
Maximum of Addis data			99.1	$\pm 7.0$	$\pm 8.4$		
Minimum of Addis data			64.4	$\pm 1.5$	$\pm 2.3$		
Probable deviation of an individual mean from mean of average			$\pm 7.7$				

\* For data from this laboratory (excluding Rehberg) mean probable deviation is  $\pm 4.6$

The results of these calculations are given in table 4.

In addition to the above experiments, in which the complete excretion curves were obtained over the maximum urine volume range, observations with ordinary urine volumes, below the augmentation limit, were made on 9 other normal subjects, and are reported, with the resulting standard clearance values, in table 3. The subjects were young men engaged in such activity as involves ordinary laboratory work.

*The augmentation limit.* It is seen from table 4, that the augmentation limit in the 8 normal subjects observed occurs at between 1.67 and 2.55 cc. per minute, if the observation made on Van Slyke in 1921 is excluded. The higher augmentation limit in this case, due to a very high average maximum clearance, falls statistically outside the group, since it differs by more than four times the mean error<sup>3</sup> from the average. There were only 4 determinations with high urine volumes in this case (data of Austin, Stillman, and Van Slyke) and they were made while the subject was about the laboratory, and not under the conditions of rest imposed on the subjects used for the analyses reported in this paper. Accordingly additional experiments on the same subject have been performed with the present series of observations, during which the subject was sitting quietly at his desk. The augmentation limit and clearances thus obtained fall within the limits of the rest of our observations. In the calculation of the average augmentation limit and maximum clearance, and the variation for the group of normal subjects, given in tables 3 and 4, only the present figures are used for this subject.

#### NORMAL VALUES AND VARIATIONS OF THE STANDARD AND MAXIMUM BLOOD UREA CLEARANCES

In table 4 are summarized the mean standard clearances of the normal subjects reported in detail in tables 2 and 3, and in addition the standard clearances calculated from previous data of Austin, Stillman and Van Slyke (1921). Similarly in table 5 are summarized

<sup>3</sup> The mean error calculated as the standard deviation,  $\pm \sqrt{\frac{\sum - \sigma^2}{n - 1}}$ , divided by the square root of the number of observations (fig. 9).

the maximum clearances of the same subjects, and in addition those on the 13 normal subjects reported by Addis (1922) with 4 or more observations on each.

For each subject, in tables 4 and 5, on whom 4 or more  $C_s$  or  $C_m$  values are available, the probable deviation of a given determination from the average in each subject, and of the mean value of each subject from the mean of all 9 subjects, have been calculated by the

formula  $0.675 \sqrt{\frac{\Sigma d^2}{n-1}}$  where  $\Sigma d^2$  represents the sum of the squares

of the deviations of individual determinations from the mean, and  $n$  represents the number of determinations. According to the theory of statistics, if the probable deviation is correctly determined half the results obtained are likely to show less deviation while half of them show greater deviations. Our number of determinations,  $n$ , is in fact too small in each individual to permit a really accurate estimate of the probable deviation of the standard or maximum clearance in his case; and likewise the number of subjects is too small to permit an accurate estimate of the probable individual deviation from the mean of the group. However, the data given, comprising 120 standard clearance determinations on 18 normal subjects and 158 maximum clearances on 20 normal subjects, appear sufficient, taken together, to yield an approximate idea of the degree of constancy to be expected in the standard and maximum clearance values in a normal individual, and of the range of these values in different normal subjects.

The "percentage probable deviation" values in tables 4 and 5 indicate that the variability among different determinations on a given subject is somewhat less for the maximum clearance than for the standard clearance. This is perhaps what could be expected, since with the smaller urine volumes, with which the standard clearance is determined, failure to empty the bladder completely must be of greater importance. We do not believe, however, that the data are sufficiently numerous to justify any comparative conclusions save that both clearances show variations of the same general order of magnitude.

The maximum clearance values calculated from Addis' 13 subjects average 82 cc. of blood cleared of urea per minute, while the values calculated from our 7 subjects and Rehberg average 75 cc. It appears

possible that the difference may be due partly to greater body size in Addis' subjects. He employs 1.82 square meters as the mean surface area (private communication through MacKay), and if the subjects

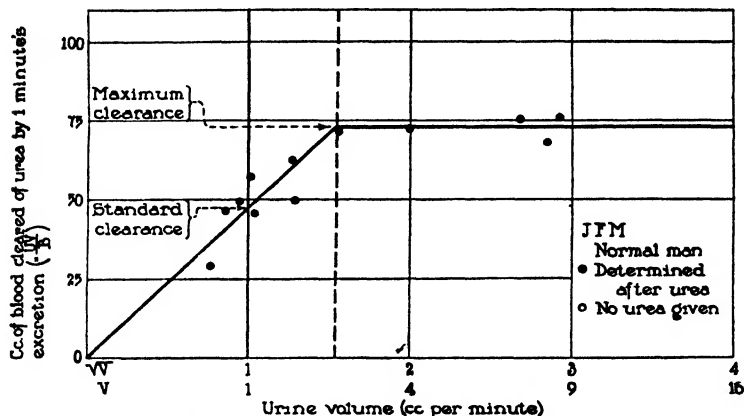


FIG. 3. UREA EXCRETION CURVE FROM NORMAL SUBJECT

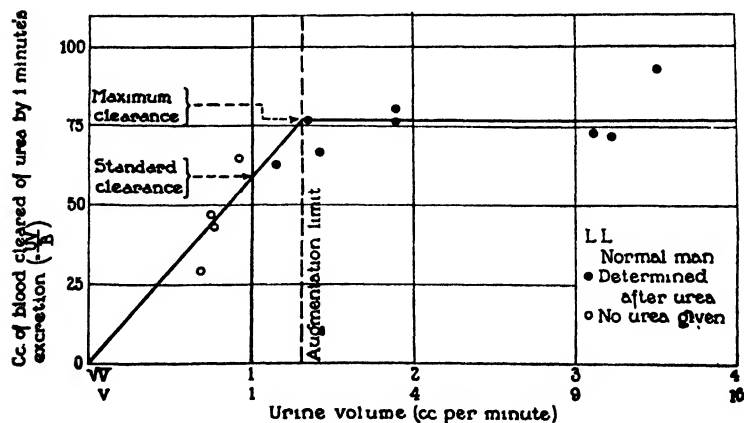


FIG. 4. UREA EXCRETION CURVE FROM NORMAL SUBJECT

he reports average as large as this, his mean  $C_e$  of 82 would correspond to one of 78 for subjects of 1.73 square meters area. As the exact heights and weights of Addis' subjects are not obtainable, we have

used our own value,  $C_m = 75$ , as the normal mean, per 1.73 square meters body area (see following paper).

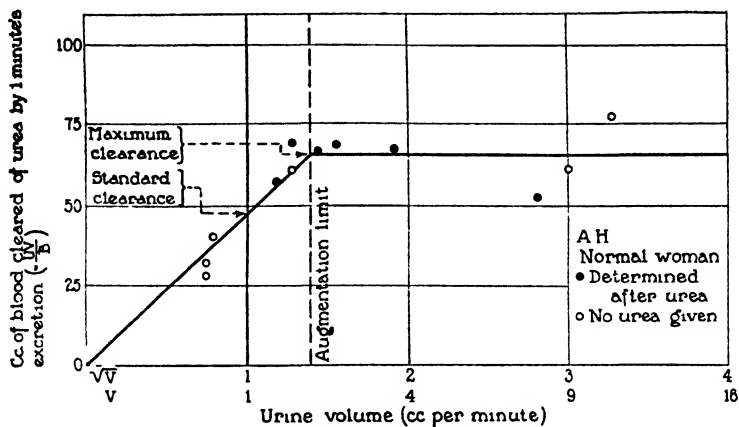


FIG. 5. UREA EXCRETION CURVE FROM NORMAL SUBJECT

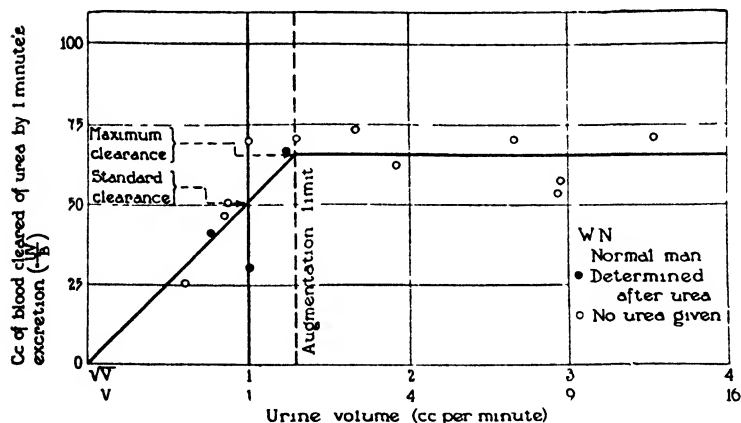


FIG. 6. UREA EXCRETION CURVE FROM NORMAL SUBJECT

The range of variation in the urea excretion curves of figures 3 to 9 covers the range of variation of the results observed in all the subjects in tables 2 and 3. The combined curves given in figures 10, 11,

and 12 therefore cover the area which, in all probability, represents the extreme variation ordinarily to be expected in normal subjects.

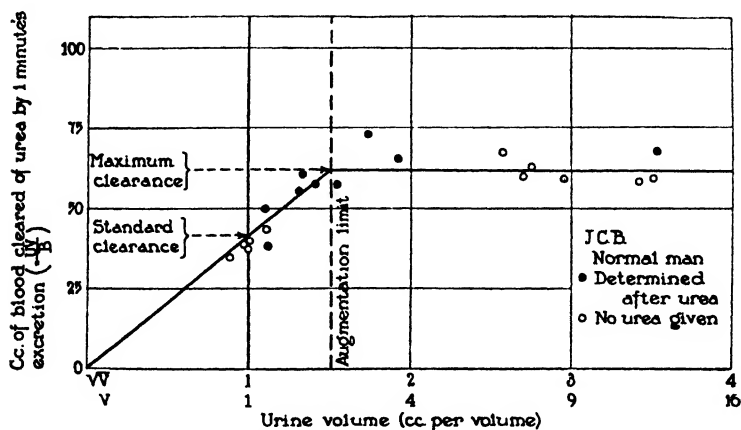


FIG. 7. UREA EXCRETION CURVE FROM NORMAL SUBJECT

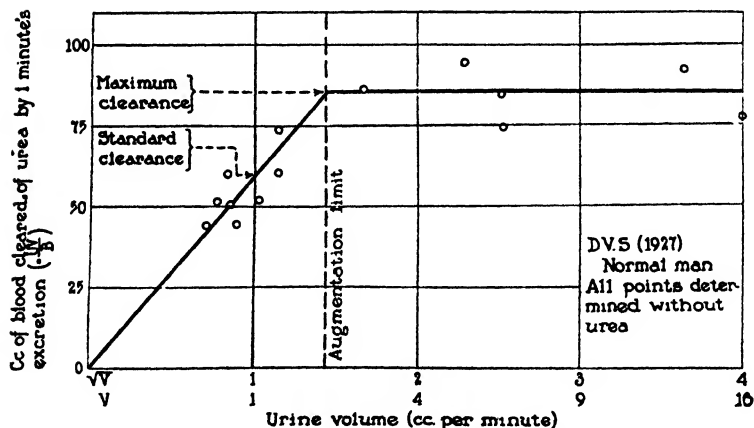


FIG. 8. UREA EXCRETION CURVE FROM NORMAL SUBJECT

The results of our experiments, plotted in figures 3 to 8, and those calculated from the data of Rehberg, plotted in figure 9, confirm the conclusions of Austin, Stillman, and Van Slyke. There is a distinct



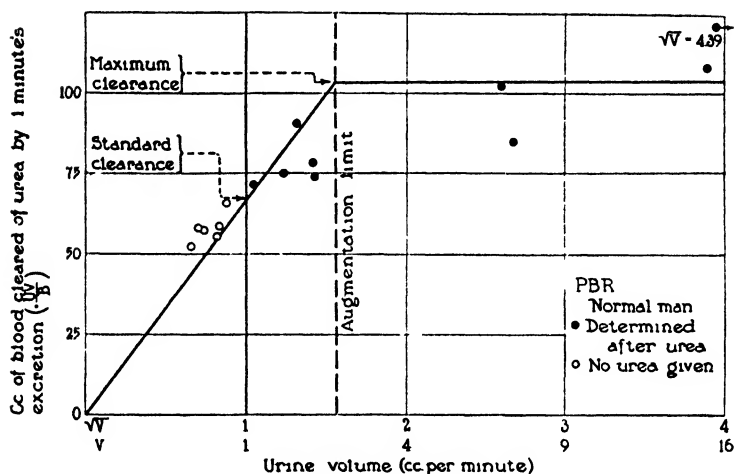


FIG. 9. UREA EXCRETION CURVE FROM NORMAL SUBJECT  
Calculated from data given by Rehberg (Biochem. J., 1926, xx, 447)

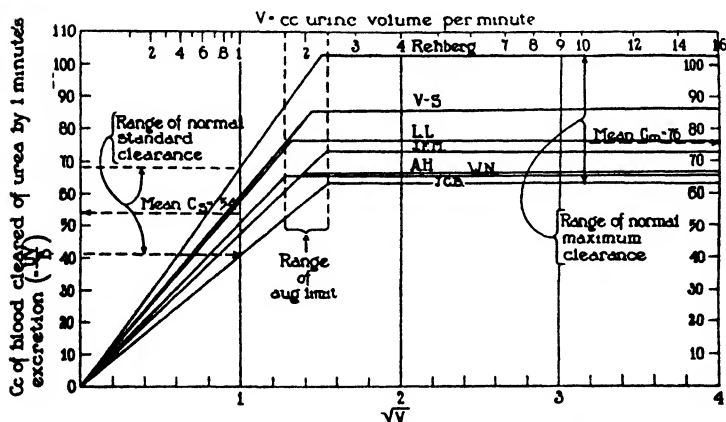


FIG. 10. RANGE OF UREA EXCRETION CURVES FOR NORMAL SUBJECTS

Only the curves shown in figures 3 to 9 are repeated, but the area enclosed by them covers also the other  $C_c$  and  $C_m$  values tabulated in tables 3 and 4. The data are uncorrected for variations in body size.

augmentation limit. Below it the clearance increases with increasing urine volume, while above it the clearance is independent of urine volume. The grouping of points about the slanting portion of the curve indicates that the square-root rule which this portion represents expresses the average effect of urine volume changes. No other line or curve could follow the experimental points more closely. The deviations of the points are frequently considerable, since other, unknown factors, besides urine volume influence the rate of urea excretion (Addis and Drury, 1923). It is obvious, however, that the

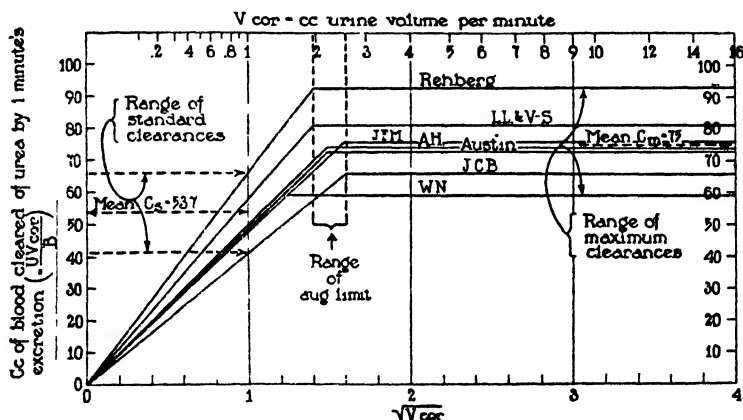


FIG. 11

Same curves as in Figure 10, but corrected for body size by multiplying  $V$  values by the correction factor  $\frac{1.73}{\text{square meters surface area}}$ .

square-root rule is followed with sufficient constancy to make interpretations of urea excretion rates with ordinary urine volumes (below the augmentation limit) much more exact when corrected for the volume effect than they would be if urine volume as a factor were neglected. In fact, the deviations of the experimental points from the slanting line representing this rule are not significantly greater than the deviations from the horizontal part of the curves covering ranges in which urine volume changes do not influence output. The square-root rule affords as satisfactory a correction for urine volume

effect, or the hydration factor of the blood which controls this effect, below the augmentation limit as appears to be obtainable.

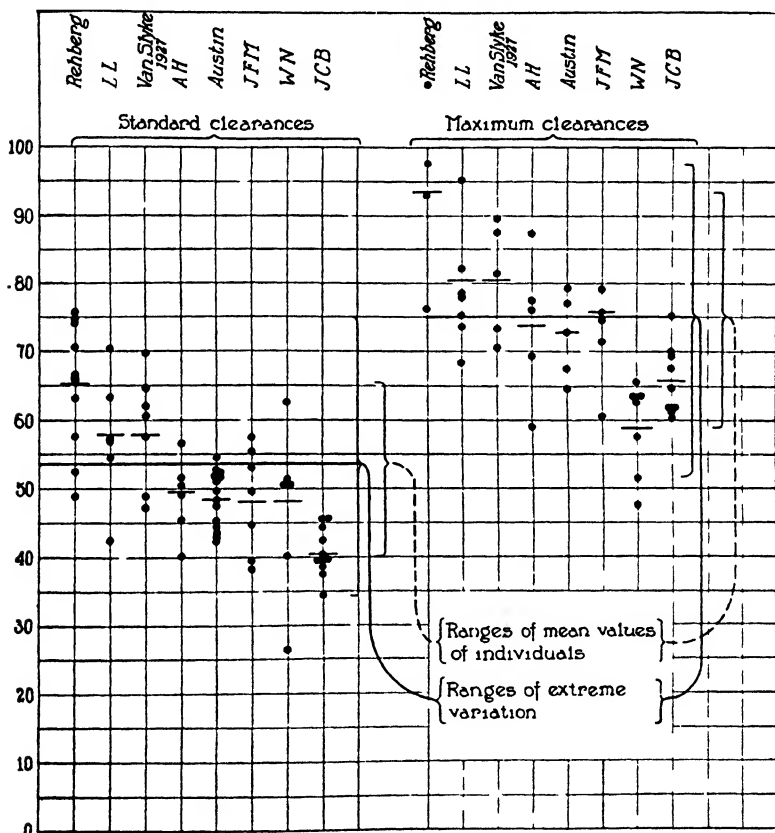


FIG. 12. VARIATIONS IN STANDARD AND MAXIMUM CLEARANCE VALUES IN NORMAL SUBJECTS

The points ● marked indicate the results of separate clearance determinations. The mean of the clearances for each subject is indicated by —.

For the increase in urea excretion rate which accompanies increase in water excretion rate up to the augmentation limit two hypothetical explanations may be offered.

1. Increase in urine volume diminishes the amount of work the kidney has to do against osmotic pressure in compressing each gram of urea from the volume it occupies in the blood to the smaller volume it occupies in the urine. Less work is required to compress the urea of 100 cc. of blood into 2 cc. of urine than to compress it further into 1 cc. of urine. The kidneys, because they work more easily with increased urine volume, may work faster, and excrete more urea per minute. From this view point, the increase in urea excretion rate which accompanies accelerated water output is a direct cause of the latter. (For quantitative calculation according to the laws of thermodynamics of the mechanical work done by the kidney per gram molecule of substance excreted see pages 93 to 96 of Barcroft (1914)).

2. The other hypothesis is that increase of renal circulation, or stimulus of the secretory activity of the renal cells, may accelerate excretion of both urea and water. The accelerated water output in this case would not be the cause of the accelerated urea output. Both would be due to a common stimulus acting on the kidney. Even dilution of the blood by water drinking might be such a cause, either inducing larger proportions of renal capillaries to open up (*vide* Richards (1920-21)) or, making the secretory cells become more active.

For the purpose of estimating from urea excretion the work which the kidneys will do under standard conditions it is, however, a matter of indifference whether the acceleration of urea output that comes with increased urine volume is caused by the latter, or merely accompanies it as the result of a renal stimulus that affects both. Whether, in introducing  $\sqrt{V}$  as a factor in the standard blood urea clearance calculation, we are dealing with the direct cause of fluctuations of clearance with urine volume, or are using urine volume as a fairly consistent indicator of such cause, does not greatly matter when we are concerned merely with measurement of renal ability.

#### SUMMARY

1. The relationship between urine volume and urea excretion has been studied in 6 more normal adults.

2. The observations of Austin, Stillman, and Van Slyke have been confirmed, that with urine volumes below a certain point (the augmentation limit) the urea output increases in direct proportion to the

square root of the urine volume, and that when the urine volume reaches this limit urea excretion attains its maximum, unaffected by further increase in urine volume.

3. The augmentation limit has been calculated for 8 normal subjects (our 6 and 2 others on whom the necessary data are found in the literature). It has been found to average 2.13 cc. of urine per minute, with a probable deviation of  $\pm 0.24$  cc.

4. When the urine volume exceeded the augmentation limit, the urea excretion was found to equal the urea content of a volume of blood which was constant for an individual within a probable variation of  $\pm 6$  per cent. This volume of blood, which a minute's excretion with urine volumes above the augmentation limit suffices to clear of urea, is termed the "*maximum clearance*." Data from this and Addis' laboratory on 20 normal adults show mean maximum clearances ranging from 64 to 99 cc. of blood per minute. For the person of average adult size (surface area = 1.73 square meters) the mean maximum clearance is about 75 cc. of blood per minute.

5. When the urine output was below the augmentation limit the square root formula of Austin, Stillman, and Van Slyke was used to estimate the urea that would be excreted with a urine volume of 1 cc. per minute, which is approximately the average output of a normal adult under ordinary conditions. The volume of blood, which 1 cc. of urine excreted in 1 minute suffices to clear of urea, is termed the "*standard clearance*," since it is estimated for a standard urine volume output. The standard clearance was found to be constant for an individual within a probable variation of  $\pm 10$  per cent. In 18 different normal adults the mean standard clearance varied from 40 to 68 cc. of blood per minute. Estimated per 1.73 square meters of surface area, the range was 41 to 65 cc., with a mean of 54 cc.

6. Line charts (figures 1 and 2) are given for graphic estimation of the maximum and standard blood urea clearances, and for comparison of the results on a percentage basis with the mean normal values.

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## STUDIES OF UREA EXCRETION.

### III. THE INFLUENCE OF BODY SIZE ON UREA OUTPUT.

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In the absence of data on subjects differing greatly in size, Austin, Stillman, and Van Slyke (1) adopted, as the simplest premise, the assumption that blood urea clearance, urine volume, and augmentation limit vary directly as the body weight.

However, Addis (8) has found that his "urea excretion ratio," which is the "maximum clearance," defined in our preceding paper (6), parallels more exactly the body surface area than it does the weight. Such a relation might be anticipated, if one considers that the rate of general metabolism, and hence probably the outputs of urea and water, parallel the body surface, and that Dreyer (3) has shown that the blood volume also parallels the surface area. The kidney weights, furthermore, were shown by Taylor, Drury, and Addis (8), to vary in rabbits in proportion to the body surface rather than to the total body weights. The clearance values observed in the animals examined before autopsy paralleled the kidney weights and skin areas. Hence these authors were led to correct their urea excretion ratios observed in patients by multiplying the observed ratios by the factor average normal surface area.

area of subject

Our experience confirms that of Addis and his colleagues. More constant normal values are obtained if one substitutes  $A$  (= surface area) in place of  $W$  in the clearance formulae. We have found it convenient to use as a unit the surface area 1.73 square meters, which is the mean of the areas of men and women of 25, estimated from the adjusted medico-actuarial tables of Baldwin and Wood published by Fiske and Crawford (5).

When corrected for body size, the formulae for standard blood urea

clearance  $C_s$ , and maximum blood urea clearance  $C_m$ , defined in the preceding paper (6), are accordingly written as

$$C_s = \frac{U}{B} \sqrt{V \times \frac{1.73}{A}} = \frac{U}{B} \sqrt{V_{\text{cor.}}}$$

$$C_m = \frac{U}{B} \times V \times \frac{1.73}{A} = \frac{U \times V_{\text{cor.}}}{B}$$

The corrected urine volume,  $V_{\text{cor.}}$ , is the observed volume of urine in cubic centimeter per minute multiplied by the factor  $\frac{1.73}{A}$ ,  $A$  being the body area in square meters that is normal for the subject's height and age. The clearance formulae, written with  $V_{\text{cor.}}$  in place of  $V$ , indicate the cubic centimeters of blood per *unit surface area* cleared of urea per minute, the unit of surface urea being 1.73 square meters. In the case of the  $C_s$  formula, with  $V_{\text{cor.}}$ , the value calculated indicates the cubic centimeters blood clearance per unit surface area when the per minute urine volume is 1 cc. per unit surface area. Blood clearance, urine volume, and hence augmentation limit are thus all based on surface area. (See derivation of original formula on page 102 of Austin, Stillman and Van Slyke (1)).

The *correction for body size* is applied as follows. The age and height of the subject having been ascertained, the value of the correction factor  $\frac{1.73}{A}$  is read from the line chart in figure 1. The observed value of  $V$ , in cubic centimeters of urine per minute, is multiplied by this factor. The corrected  $V$  thus obtained is used in the standard clearance formula,  $C_s = \frac{U \sqrt{V_{\text{cor.}}}}{B}$ , or the maximum clearance formula,

$$C_m = \frac{UV_{\text{cor.}}}{B}, \text{ for the calculations outlined in the preceding paper (6).}$$

In the correction factor  $\frac{1.73}{A}$ ,  $A$  represents in square meters the mean surface area of normal persons of the subject's height and age. Surface area is thus used as the nearest available parallel to the mass of functioning renal tissue (8) present in a normal subject. Because of the likelihood that the subjects examined will be obese, edematous, or

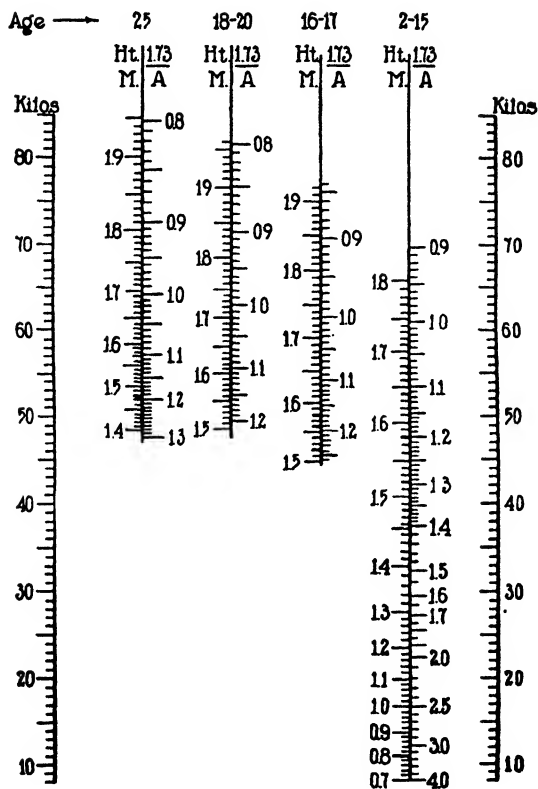


FIG. 1. CHART FOR ESTIMATING VALUES OF THE CORRECTION FACTORS,  $\frac{1.73}{A}$ , FROM HEIGHT AND AGE, AND FOR COMPARING OBSERVED WEIGHTS WITH WEIGHTS NORMAL FOR THE SUBJECTS EXAMINED

The value of  $\frac{1.73}{A}$  is read off opposite the height of the subject in meters, on the scale for subjects of his age, or of 25 if he is mature. A horizontal line from the same point to the weight scale on either side cuts the latter at a point indicating the ideal weight of the subject.<sup>1</sup>

<sup>1</sup>The ideal weight is not used in calculation of the clearance corrections, but we have added the "ideal weight" scales to Fig. 1 because we have found them of convenience for comparison with the observed weights of edematous, obese, or emaciated patients.

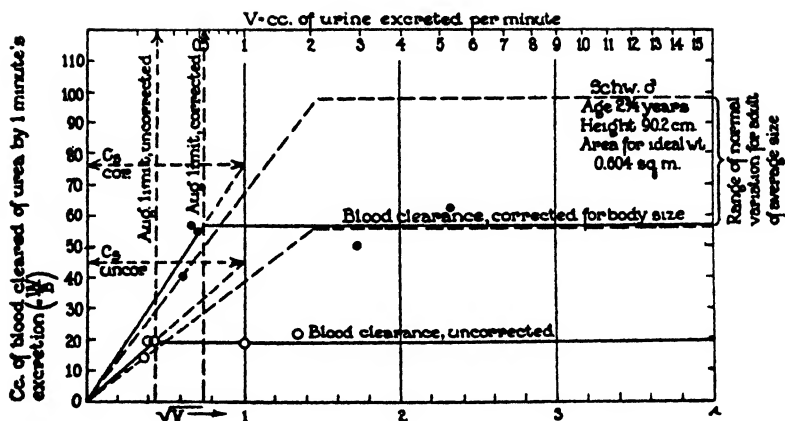


FIG. 2. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 13.6 KG. IDEAL WEIGHT

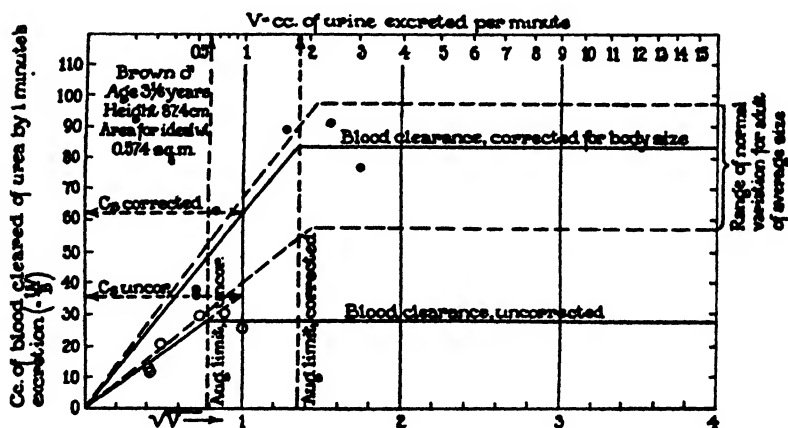


FIG. 3. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 12.6 KG. IDEAL WEIGHT

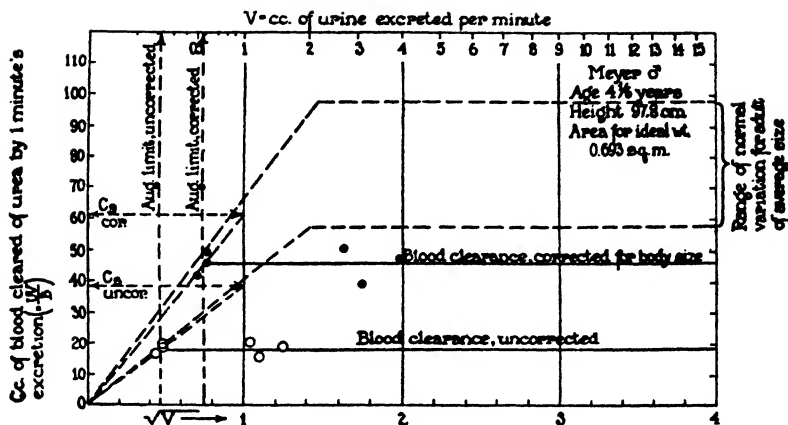


FIG. 4. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 15.4 KG. IDEAL WEIGHT

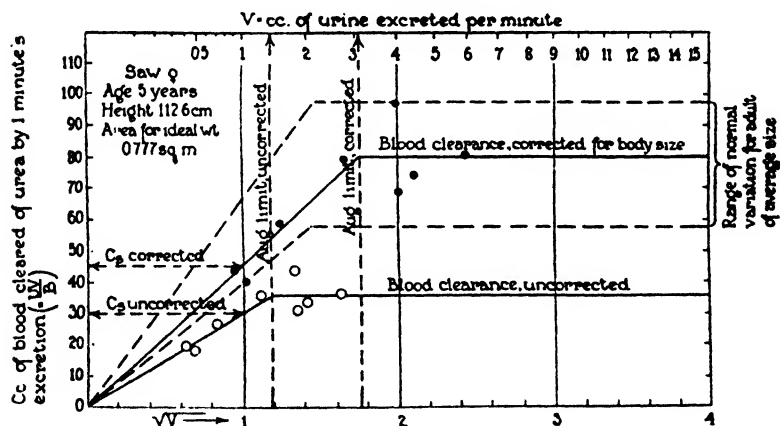


FIG. 5. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 19.3 KG. IDEAL WEIGHT

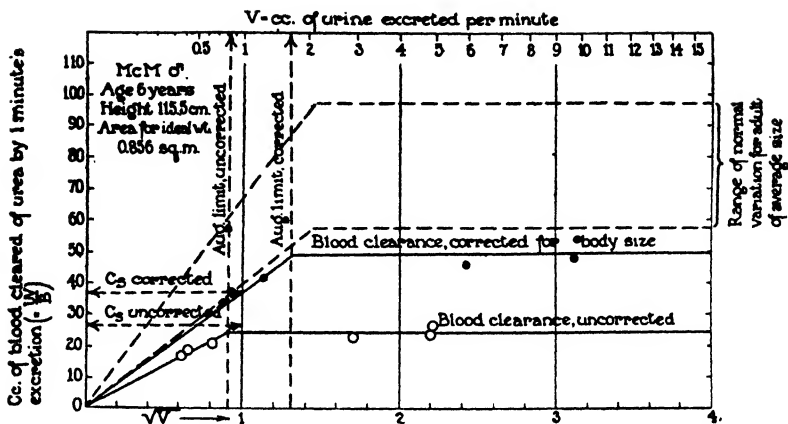


FIG. 6. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 21.1 KG. IDEAL WEIGHT

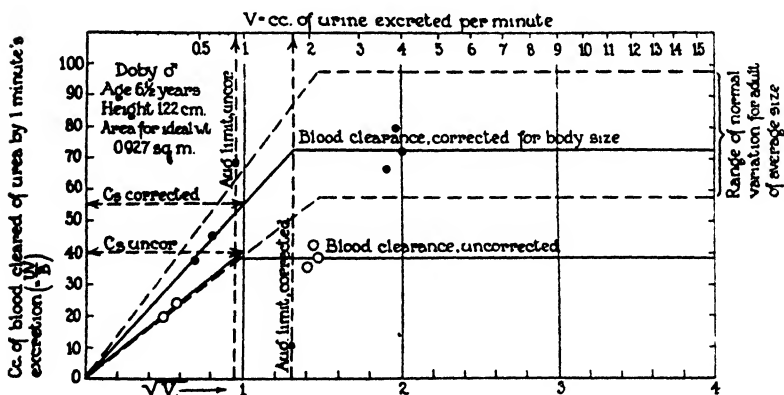


FIG. 7. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 23.8 KG. IDEAL WEIGHT

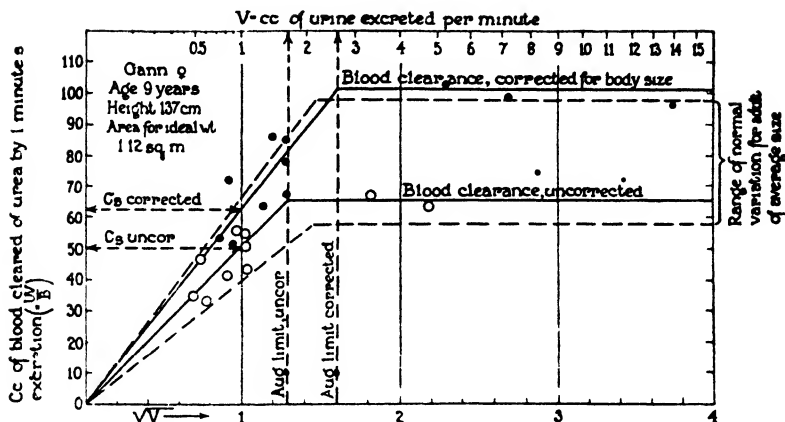


FIG. 8. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 31.8 KG. IDEAL WEIGHT

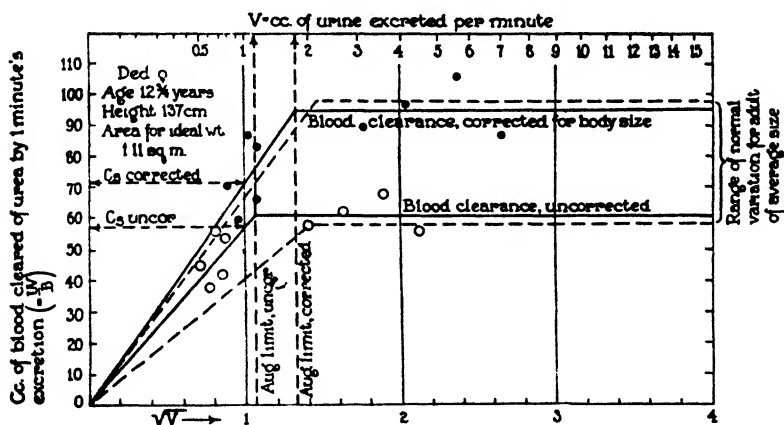


FIG. 9. UREA EXCRETION CURVE, UNCORRECTED AND CORRECTED FOR SURFACE AREA, OF CHILD OF 31.5 KG. IDEAL WEIGHT

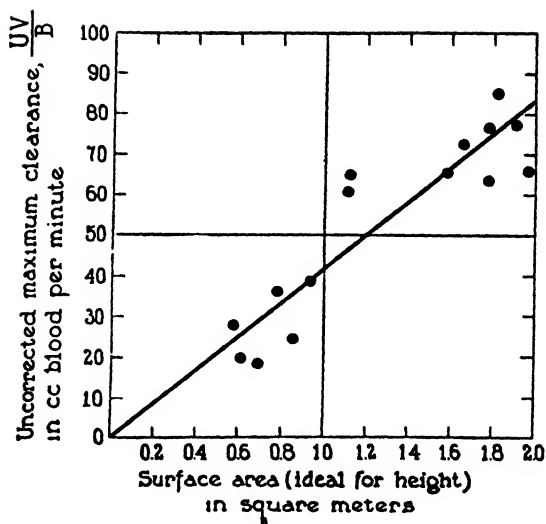


FIG. 10. RELATIONSHIP OF SURFACE AREA TO UNCORRECTED MAXIMUM CLEARANCE VALUES OF NORMAL ADULTS AND CHILDREN

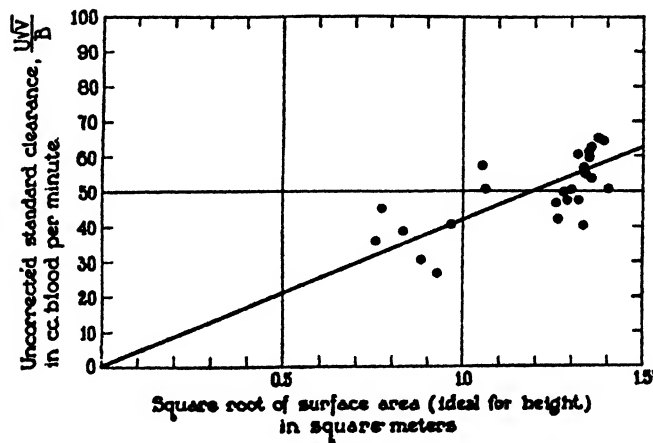


FIG. 11. RELATIONSHIP OF SURFACE AREA TO UNCORRECTED STANDARD CLEARANCE VALUES OF NORMAL ADULTS AND CHILDREN



emaciated, it appears more exact, for correction of the clearance values, to use the body surface estimated from the weight which is ideal for a subject of that height and age, rather than the actual surface estimated from the height and the observed weight at the moment of observation. For adults over 25 years of age we use as  $A$  the surface area of the average normal person of 25 and the subject's height.

Figure 1 has been constructed as follows. From the tables of Baldwin and Wood (5) curves were constructed, with weight and height as the coördinates, for males and females of different ages from 5 to 25. From 5 to 15 the height-weight relationship could be expressed by a single curve, but the increasing weight-height ratio during adolescence necessitated 2 additional curves for the age interval 16-25. From the height-weight values of these curves, surface areas were calculated by Du Bois' (4) height-weight formula, and a second set of curves was constructed representing mean normal height-surface area relationships for the ages covered. From these two sets of curves the scales of figure 1 were constructed, down to those for the height 1.4 meter (age about 12 years). Below this body size we have employed the data of Benedict and Talbot (2) as given in their figures 7, 8, 10, and 13, which give height-weight and height-area relationships down nearly to birth. In constructing the scales of figure 1 we have used the means of the height-weight and height-area relationships of males and females. The sexes differ too little to justify, for our purpose, separate scales for each.

The standard clearance is affected by a given deviation from ordinary body size only in proportion to the deviation of  $\frac{\sqrt{1.73}}{A}$  from unity, since the  $V$  value by which the factor is multiplied is under the square root sign in the formula  $C_s = \frac{U}{B} \sqrt{V}$ . Hence a variation of  $\pm 10$  per cent in surface area affects the  $C_s$  by only approximately  $\pm 5$  per cent. Such a variation corresponds to a height range in adults between 157 and 181 cm., or 62 and 71 inches. In determining the standard clearance for clinical diagnosis a factor of less than 5 per cent can be neglected; and the correction factor need be applied only to patients outside the above limits of body size.

TABLE I  
Data concerning urea excretion

Subject	U Urine urea nitrogen	B Blood urea nitrogen	V Urine volume	$V \times \frac{1.73}{\text{Area}}$ Urine volume corrected for body size	Uncorrected clearances		Clearances corrected for body size	
					$\frac{UV}{B}$ Observed clearance*	$\frac{U\sqrt{V}}{B}$ Standard clear- ance calculated for $V = 1$ from observations below augmenta- tion limit	$\frac{U(V \times \frac{1.73}{A})}{B}$ Observed clear- ance*	$\frac{U\sqrt{V \times \frac{1.73}{A}}}{B}$ Standard clearance calculated for $V \times \frac{1.73}{A} = 1$ from observations below augmenta- tion limit
Schw. ♂ 2 $\frac{1}{2}$ years. 15.9 kgm. 90.2 cm. height 0.620 sq. m. surface area ideal for height $\frac{1.73}{\text{Area}} = 2.80$	mgm per 100 cc	mgm per 100 cc	cc per minute	cc per minute	cc blood per minute	cc blood per minute	cc blood per minute	cc blood per minute
	1,121	(10.5)	0.132	0.370	14.1	38.8	39.5	64.9
	1,346	10.5	0.154	0.431	19.8	50.4	55.3	84.2
	1,147	10.5	0.176	0.493	19.2	45.8	53.8	76.5
	260	15.1	1.03	0.287	17.7*		49.4*	
	175	(15.0)	1.88	0.529	21.9*		61.7*	
Brown. ♂ 3 $\frac{1}{2}$ years. 14.6 kgm. 87.4 cm. height. 0.595 sq. m. surface area ideal for height $\frac{1.73}{\text{Area}} = 2.91$	Average clearance				19.8*	45.0	55.5*	75.2
	832	11.0	0.167	0.486	12.6	30.9	36.7	52.6
	756	(10.6)	0.167	0.486	11.9	29.2	34.7	49.7
	912	10.2	0.233	0.677	20.8	43.2	60.6	73.5
	527	9.5	0.534	1.55	29.5	40.4	86.0	69.0
	337	8.9	0.793	2.4	30.2*		90.8*	
	235	(9.20)	1.000	2.91	25.5*		74.4*	
	Average clearance				27.9*	35.9	82.6*	61.2

Meyer, $\sigma^2$ 4 $\frac{1}{4}$ years. 17.9 kgm. 97.8 cm. height. 0.675 sq. m. surface area ideal for height $\frac{1.73}{\text{Area}} = 2.53$	1,067	12 7	0 197	0 498	16 6	37 3	41 8	59 1
	1,088	13 1	0 233	0 59	19 7	40 7	49 0	63 7
	1,033	13 1	0 233	0 59	18 4	38 1	46 5	60 5
	286	15 2	1 08	2 73	20 3*		51 4*	
	194	15 1	1 22	3 09	15 7*		39 7*	
	184	(15 2)	1 57	3 97	19 0*		48 1*	
Average clearance								
Saw, $\sigma$ 5 years. 24.8 kgm. 112.6 cm. height 0.815 sq. m surface area ideal for height 1 73 $\frac{\text{Area}}{\text{Area}} = 2.12$	562	11 4	0 400	0 848	19 7	31 2	41 8	45 4
	433	11 3	0 472	1 00	18 1	26 3	38 3	38 3
	406	10 7	0 693	1 47	26 3	31 6	55 8	46 0
	294	(10 2)	1 23	2 61	35 5	31 9	78 2	48 4
	262	10 7	1 78	3 77	43 5*		92 3*	
McM $\sigma^2$ 6 years. 17.5 kgm. 115.5 cm. height. 0.850 sq. m. surface area ideal for height 1 73 $\frac{\text{Area}}{\text{Area}} = 2.04$	169	10 0	1 82	3 86	30 8*		65 2*	
	171	(10 3)	2 00	4 24	33 3*		70 4*	
	152	11 2	2 67	5 66	36 2*		76 8*	
	Average clearance..							
	636	14 5	0 383	0 781	36 0*	30 3	76 2*	44 5
	612	14 3	0 433	0 883	16 8	27 2	34 3	38 8
	452	14 4	0 650	1 327	18 5	28 2	37 8	40 5
	124	16 0	2 92	5 96	20 4	25 3	41 6	36 1
	83 9	17.2	4 83	9 86	22 6*		46 2*	
	88 7	16 3	4 88	9 96	23 6*		47.8*	
					26 5*		54 2*	
Average clearance..								
					24 2*	26 9	49.4*	38 5

TABLE 1—Continued

Subject	U Urine urea nitrogen	B Blood urea nitrogen	V Urine volume	$V \times \frac{1.73}{\text{Area}}$ Urine volume corrected for body size	Uncorrected clearances		Clearances corrected for body size	
					$\frac{U}{V}$ Observed clear- ance*	$\frac{U \sqrt{V}}{B}$ Standard clear- ance calculated for $V = 1$ from observations below augmen- tation limit	$\frac{U \left( V \times \frac{1.73}{A} \right)}{B}$ Observed clear- ance*	$\frac{U \sqrt{V \times \frac{1.73}{A}}}{B}$ Standard clearance calculated for $V \times \frac{1.73}{A} = 1$ from observations below augmen- tation limit
Doty. ♂ 6 $\frac{1}{2}$ years. 21.3 kgm. 122.0 cm. height. 0.927 sq. m. surface area ideal for height 1.73 = 1.87 Area	mgm. per 100 cc.	mgm. per 100 cc.	cc. per minute	cc. per minute	cc. blood per minute	cc. blood per minute	cc. blood per minute	cc. blood per minute
	1,435	18.0	0 250	0 467	19.9	39.9	37.2	54.5
	1,270	17.9	0 343	0 640	24.3	41.6	45.4	56.8
	307	17.0	1.97	3.68	35.6*		66.5*	
	281	13.8	2.08	3.88	42.4*		79.2*	
	303	17.0	2.17	4.05	38.7*		72.3*	
Average clearance.....					38.9*	40.8	72.7*	55.7
Gann. ♀ 9 years. 26.8 kgm. 137 cm. height. 1.12 sq. m. surface area ideal for height $\frac{1.73}{\text{Area}} = 1.55$	937	(13.2)	0 486	0 751	34.5	49.5	53.3	61.6
	1,115	13.2	0 548	0 846	46.3	62.6	71.5	77.8
	653	11.7	0 594	0 918	33.2	43.0	51.3	53.5
	466	9.4	0 833	1.29	41.3	45.2	63.8	56.2
	488	8.2	0 933	1.44	55.5	57.5	85.8	71.5
	453	8.8	1.07	1.65	54.9	53.2	84.8	66.2
	435	9.2	1.07	1.65	50.4	48.9	77.8	60.8
	479	11.9	1.08	1.67	43.6	41.8	67.4	52.0
	273	14.0	3.40	5.25	66.3*		102.5*	
	197	14.5	4.69	7.24	63.8*		98.6*	
Average clearance.....					65.1*	50.2	100.6*	62.5

Ded. 9	1,013	11 3	0 500	0 775	44 9	63 4	69 6	79 0
12 $\frac{1}{2}$ year.	739	11 4	0 583	0 904	37 8	49 5	58 6	61 5
32.5 kgm.	866	10 4	0 667	1 034	55 6	62 1	86 0	83 4
137 cm. height.	556	9 7	0 733	1 136	42 0	49 1	65 2	61 1
112 sq. m. surface area	760	10 7	0 750	1 163	53 2	61 5	82 6	76 5
ideal for height	274	9 6	2 00	3 10	57 2*		88 5*	
$\frac{1.73}{Area} = 1.55$	248	10 7	2 67	3 98	62 0*		92 3*	
	189	(10 0)	3 58	5 55	67 7*		105 0*	
	122	9 9	4 50	6 97	55 8*		85 9*	
Average clearance.					60 7*	57 1	92 9*	72 3

\* Figures marked \* represent maximum clearance values, observed when  $V$  was above the augmentation limit for the subject.

In estimating the maximum blood urea clearance, however, by the formula  $C_m = \frac{UV}{B}$ , a 10 per cent correction to  $V$  causes a 10 per cent correction to the  $C_m$  value calculated. Hence, to avoid an error greater than  $\pm 5$  per cent, we can neglect body size in estimating the maximum clearance only in adults between 164 and 176 cm., or 65 and 69 inches, in height.

### EXPERIMENTAL

In order to measure satisfactorily the influence of body size on urea excretion rate it is necessary to compare children with adults. With ordinary adults variation in size, as a factor in influencing the volume of blood, the urea content of which is excreted per minute, is less important than other, unknown factors, which may be summarized as "individual constitution." These may cause the standard or maximum clearance of an individual, of average size and normal to all appearances, to vary by as much as 25 per cent from the mean normal clearance. The effect of body size is so obscured by the greater effects of individual constitution that the size effect in adults can be measured only by statistical methods. In order to make it an outstanding factor it is necessary to study subjects with a greater size range than can be obtained in the adults usually available for observation.

We have accordingly, by the technique described in the preceding paper (6), determined the urea excretion curves on a number of children. The numerical data are given in table 1, and the curves in figures 2 to 9. The correction for body size is made, as previously described, by multiplying the observed  $V$  value by the factor  $\frac{1.73}{A}$ .

### DISCUSSION OF RESULTS

It is obvious from figures 2, 3, 4, 5, 6, 7, 8, and 9, that correcting the blood urea clearances, by multiplying the observed values of  $V$  in cc. urine excreted per minute by the factor  $\frac{\text{average adult surface area}}{\text{surface area of subject}} = \frac{1.73}{A}$ , causes data from children, at least down to 3 years of age, to fall

within the same range as the data of adults in respect to maximum blood urea clearance and standard blood urea clearance. This manner of correcting for body size therefore makes it possible to put on a common basis for comparison, with regard to urea excreting ability, subjects of the widest range of body size that is likely to be encountered in this type of examination.

The figures for the augmentation limit, corrected for body size, show an average of 1.7 cc. per minute. This is somewhat lower than the corresponding figure for normal adults (2.1 cc.), but within the limit of variation for adults. The augmentation limit values, show a greater individual variation for children than for adults. This may be due to the fact, that, with the small urine volumes often found in determinations of the standard clearance in children, failure to empty the bladder completely, and consequent errors in measuring the urine volume, become of increased importance. For routine determinations of the urea excreting function in small children it may therefore prove advantageous when possible to give sufficient fluids to cause free diuresis, and determine the maximum instead of the standard clearance.

In a recent paper (7) Rabinowitch and Breitman have given for a number of children data from which the standard clearance (corrected for surface area calculated from the observed weight) can be calculated. The experimental procedure of these authors is such that their results can not be very accurately compared with ours. First, they give a large amount of urea just before the test, which causes the blood urea to vary irregularly. Second, they use as the blood urea value the mean of the two figures obtained one hour before, and shortly after the 1-hour period from which the urine urea is determined. Third, they determine the urea output during only a single 1-hour period on each child. However, if their data are recalculated in such a way that the second blood urea value only is used, and the results are plotted into our figure 11, the axis of their group falls near to that for our 25 individuals. That the dispersion for their results is greater than for ours is probably due to the factors mentioned above.

It does not seem necessary to present comparative calculations to show that the area correction is more exact than the weight correction. Such calculations may readily be made from the data in table 1. They show that the weight formulae,  $\frac{U}{B} \sqrt{\frac{V}{W}}$  and  $\frac{UV}{BW}$  yield in the children values for the standard and maximum clearance respectively which are quite above the range for adults: urea excretion rates in small sub-

jects do not decrease so rapidly as body weights. They decrease rather as the surface area, or the  $2/3$  power of the weight.

The degree of exactness with which the maximum clearance,  $\frac{UV}{B}$ , varies in proportion to surface area in different subjects is indicated by figure 10 in which we have plotted against surface area the mean maximum clearance for each of the 7 adults reported from this laboratory in the preceding paper (6), and each of the children reported in the present paper. In figure 11 the uncorrected standard clearance,  $\frac{U \sqrt{V}}{B}$ , is similarly plotted against the square root of surface area for each of the 17 adults reported in the preceding paper, and each of the children in the present one.

#### SUMMARY

The calculated maximum and standard blood urea clearances, previously defined (6), may be corrected for variations in body size by means of a factor based on the assumption, introduced by Addis (8), that excretion varies directly as surface area. Thus corrected, data from small children yield the same normal values as adults for the maximum and standard clearances, and also for the augmentation limit of urine volume, at which maximum excretory efficiency is attained.

The nature of the standard clearance formula is such that correction for body size in persons between 62 and 71 inches in height does not exceed 5 per cent, and in tests of renal function may be neglected.

For the maximum clearance the range of height with less than 5 per cent correction is 65 to 69 inches.

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## STUDIES OF UREA EXCRETION.

### IV. RELATIONSHIP BETWEEN URINE VOLUME AND RATE OF UREA EXCRETION BY PATIENTS WITH BRIGHT'S DISEASE.

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(Received for publication, August 21, 1928.)

Previous papers from this hospital (2, 6) have shown that until the urine volume reaches a certain augmentation limit, averaging about 2 cc. per minute, the rate of urea excretion by a normal man increases in direct proportion to the square root of the urine volume, but that when the urine volume attains this limit urea excretion attains its maximum, equal per minute to the urea content of 60 to 90 cc. of blood, and is unaffected by further increase in urine volume.

In order to ascertain the manner in which these relationships are affected in Bright's disease we have determined the hourly blood urea clearances (defined in a preceding paper (6)) of a number of patients with this disease in whom maximum changes in urine volumes were induced by regulating the water intake. Results from six typical cases are reported in this paper. The experimental technique was identical with that outlined in the paper on normal subjects (6). In each case experiments on two or more days were performed in order to obtain the desired amount of data, but the days were sufficiently close together, and the condition of each patient sufficiently stable, to permit a fair degree of assurance that the renal function in each subject was practically unchanged during the period.

In most of the experiments on patients Jac., Cic., Val., and Wol., cutaneous blood was taken and the urea content estimated with the micro-gasometric method of Van Slyke (7), while in some of the shorter experiments on these four patients and in all the experiments on patients Chi. and Gia. venous blood was drawn and its urea content estimated with the aeration method of Van Slyke and Cullen (8).

In two cases, patients Val. and Wol., it was impossible to get urine

TABLE 1  
Data of cases

Case	Blood pressure	Size of heart	Eye grounds	Hemoglobin as capacity	Red blood corpuscles	White blood corpuscles	Plasma proteins				Blood urea	Plasma creatinine	Plasma non-protein N	Urine protein (Ksach)	Diuresis (ca.)	Specific gravity	Sediment	Phenolphthalein test in two hours	Mean standard blood $\frac{U}{V}$ urea, clearance corrected to body size
							Albumin	Globulin	Total protein	A/G ratio									
1 Ckl. Hospital No. 5335	100/70	Normal	Normal	13.2	4.98	5,500	3.34	2.32	5.66	1.44	15	1.3	34	0.3	1,000	1013-1017	+++ RBC, + hyaline and granular casts, no DRG*	70	62
2 Jac. Hospital No. 5699	134/85	Slightly increased	Normal	20.2	5.31	6,100	1.71	2.11	3.82	0.81	20	1.9	41	4	1,000	1012-1018	+ RBC, + WBC, +++ hyaline casts, ++ granular casts, + DRG	22	22
3 Ck. Hospital No. 5644	115/80	Normal	Normal	20.1	4.50	8,900	1.70	2.24	3.94	0.76	22	1.9	29	6	1,200	1010-1014	+ RBC, + WBC, +++ hyaline casts, + granular casts, + DRG	35	18
4 Val. Hospital No. 5446	145/75	Somewhat increased	Normal	12.0	4.39	7,500	2.11	1.92	4.03	1.10	40	3.0	32	6	1,600	1010-1018	+++ RBC, ++ WBC, ++ RBC casts, ++ granular casts, no DRG		

5	120/75	Normal	Nor- mal	14.0	3.11	8,200	2.41	2.43	4.84	0.99	80	5.1	87	ca. 1	1,600/1010- 1016	++ RBC, ++ WBC, + hyaline casts, + granular casts, no DRG
6	145/90	Normal	Nor- mal	14.2	3.97	6,200	3.87	2.57	6.44	1.50	55	2.4	62	2	1,400/1006- 1010	+ RBC, ++ WBC, + hyaline casts, + granular casts, no DRG

\* DRG = double refractive globules.

volumes as low and as high respectively as desired, although many attempts on different days were made. This was due to the loss of the power of concentration and of dilution respectively in these two patients.

The laboratory findings in our 6 cases of Bright's disease are given in table 1. The terms of classification are those used by Addis (1).

# CASE HISTORIES

*Case 1. Chi.* Hospital No. 5335. Boy, 13 years old. When 7 years old he had acute glomerulonephritis, now relapse with hematuria and some edema.

TABLE 2  
*Correction factors for body size*

Case			Age	Weight	Height	Body surface area observed	Weight ideal for height and age	Area ideal for height and age	Correction factor	
Name	Number	Hospital number							$\frac{1.73}{\text{Area observed}}$	$\frac{1.73}{\text{Area ideal}}$
			<i>years</i>	<i>kgm.</i>	<i>cm.</i>	<i>sq. m.</i>	<i>kgm.</i>	<i>sq. m.</i>		
Chi.	1	5335	13	38	145.9	1.30*	37.5	1.29*	1.33	1.34
Jas.	2	5699	24	56	173.0	1.66	66.8	1.79	1.04	0.97
Cic.	3	5644	24	48	163.4	1.50	59.8	1.65	1.15	1.05
Val.	4	5446	24	59	176.0	1.73	69.2	1.84	1.00	0.94
Gia.	5	5388	24	64	175.0	1.79	68.3	1.83	0.97	0.95
Wol.	6	5731	16	38	155.0	1.31	46.9	1.43	1.32	1.21

\* Calculated from the table of Benedict and Talbot for children: Carnegie Trust Wash. Publ. No. 302, 1921, p. 61.

Surface areas of other patients are calculated by Du Bois' formula.

No loss of ability to excrete urea or phthalein. *Course of the disease:* After 6 weeks sent home with no edema, only a trace of albuminuria, and a slight microscopic hematuria. Seen 6 months and one year later, when the hematuria had quite disappeared, while the slight albuminuria persisted. Other findings normal.

*Case 2. Jac.* Hospital No. 5699. Man, 24 years old. One year ago, tonsillitis followed by albuminuria and marked edema. This cleared up gradually in 6 months, but after chrysarobin treatment for psoriasis severe relapse set in with edema, ascites and hydrothoras. *Course of the disease:* Edema and anasarca cleared up completely in one month. Seen 6 months later, there was then no edema and only a few red cells and casts in the urine.

*Case 3. Cic.* Hospital No. 5644. Man, 24 years old. Syphilis found 6 years ago, since then repeated treatment with salvarsan and mercury. One

year ago edema and ascites began, both still present. Renal function diminished. Wassermann reaction negative. *Course of the disease:* Edema nearly disappeared after 3 months. Seen 6 months later, when the condition was unchanged as at discharge from hospital.

*Case 4. Val.* Hospital No. 5446. Man, 24 years old. The last year some edema, hematuria and dyspnea on exertion have been present. Now only slight edema. Diminished ability to excrete urea and phthalein. *Course of the disease:* After 3 months, hematuria much decreased and the blood hemoglobin content somewhat increased. Seen 3 months later, when the condition was unchanged.

*Case 5. Gia.* Hospital No. 5338. Man, 24 years old. Tonsillitis, bronchitis and pleurisy 8 months ago, followed by marked edema, ascites, oliguria and hematuria. Now no ascites, only slight edema, no hematuria; but dyspnea on exertion. Marked loss of renal function. Unusual normal blood pressure and normal heart, despite advanced renal disease and function loss. *Course of the disease:* Died 9 months later in uremia. Histological diagnosis: Chronic glomerulonephritis.

*Case 6. Wol.* Hospital No. 5731. Scarlet fever 6 years ago. During the convalescence albuminuria appeared and has been present since then. 10 months ago, moderate edema, nausea and vomiting set in and hypertension was found. Since then the condition has somewhat improved. On admission only a trace of edema and a little nausea were present. Phthalein excretion in 2 hours was 25 to 31 per cent. *Course of the disease:* During 3 months, nausea and edema disappeared. Five months later the patient was re-examined. Although he was subjectively well, progress towards uremia was indicated by a blood creatinine of 4.8 mgm. per cent and a blood urea nitrogen of 56 mgm. The data recorded in table 3 were obtained 6 weeks after the first admission.

#### RESULTS AND DISCUSSION

The results are given in table 3 and in figures 1 to 6. The clearance values are corrected for body size, by calculating them from observed  $V$  values multiplied by the factor  $\frac{1.73}{\text{sq. m. ideal area}}$ , as described in the preceding paper (6). The correction factors are given in table 2.

As in the normal subjects reported in the foregoing paper, the same effect of a given urine volume on urea excretion was observed, whether the volume had been reached through increase or decrease.

For 5 of the 6 patients the position of the augmentation limit has

TABLE 3  
Data concerning urea excretion

	Time	V Urine volume	V cor. Urine volume corrected for body size by factor $\frac{1.73}{\text{Area}}$	U Urine urea nitrogen	B Blood urea nitrogen	$\frac{UV \text{ cor.}}{B}$ Observed clearance*	$\frac{U \sqrt{V} \text{ cor.}}{B}$ Calculated stand- ard and below clearance (for V) augmentation limit	Per cent of average normal clearance
		cc. per minute	cc. per minute	mgm. per 100 cc.	mgm. per 100 cc.	cc. blood per minute	cc. blood per minute	per cent
Exp. No. A 12. Chi. 8:50 a.m., 100 cc. of water. Venous blood	9-11	0.97	1.30	413	7.5	71.6	62.7	116
Exp. No. A 13. Chi. 8:40 a.m., 100 cc. of water. Venous blood	9-11	0.67	0.90	678	10.1	60.0	63.6	118
Exp. No. A 14. Chi. 8:20 a.m., 100 cc. of water. Venous blood	9-11	0.41	0.55	1019	11.9	96.9	63.4	117
Exp. No. A 15. Chi. 8:20 a.m., 100 cc. of water. Venous blood	9-11	0.21	0.28	966	11.1	24.4	46.2	85
Exp. No. A 16. Chi. 8:20 a.m., 100 cc. of water. Venous blood	9-11	0.30	0.40	828	7.0	47.6	75.0	139
Exp. No. A 17. Chi. 8:20 a.m., 100 cc. of water. Venous blood	9-11	0.28	0.38	1005	10.1	37.4	61.0	113
Exp. No. A 18. Chi. 6 a.m., 20 grams urea and 300 cc. of water. 7, 8, 9, 10, and 11 a.m., 300 cc. of water each time. Venous blood	9-10 10-11 11-12	6.50 6.92 4.50	8.71 9.27 6.03	282 240 336	30.4 27.0 25.1	80.9* 82.4* 80.7*		108* 110* 108*





TABLE 3—Continued

Time	V Urine volume cc per minute	V cor Urine volume corrected for body size by factor $\frac{1.73}{\text{Area}}$ from table 2	U Urine urea nitrogen mgm per 100 cc	B Blood urea nitrogen mgm per 100 cc	$\frac{UV \text{ cor}}{B}$ Observed clearance*	$\frac{U \sqrt{V} \text{ cor}}{B}$ Calculated stand and below clearance (for V) augmentation limit	Per cent of average normal clearance
		cc per minute	mgm per 100 cc	mgm per 100 cc	cc blood per minute	cc blood per minute	per cent
Exp 20-a. Cic.	10-11	0 37	384	16 2	9 1	14 8	27
9 a.m., 100 cc. of water. Venous blood	11-12	0 60	386		15 0	19 0	35
Exp 20-b. Cic.	10-11	0 50	347	15 2	11 9	16 6	31
9 a.m., 100 cc. of water. Venous blood	11-12	0 35	270		6 6	10 8	20
Exp 20-c. Cic.	10-11	1 27	198	12 1	21 7	18 9	35
9 a.m., 100 cc. of water. Venous blood	11-12	0 92	299		23 8	24 3	45
Exp. No. 22. Cic.	9-10	1 92	354	36 6	19 4*		26*
7:30 a.m., breakfast with 15 grams urea	10-11	1 87	344	35 1	19 2*		26*
and 1000 cc. of water. 11:50 a.m.,	11-12	1 83	345	34 5	19 2*		26*
lunch with 1000 cc. of water. 1:20	12-1	2 17	349	34 6	22 9*		31*
and 2:05 p.m., 500 cc. of water each	1-2	4 17	209	37 0	24 7*		33*
time. Cutaneous blood	2-3	4 50	230	35 4	30 7*		41*
	3-4	4 83	185	33 8	27 8*		37*
Exp. No. 14. Val.	9-10	1 10	244	21 8	11 6	11 3	21
8 a.m., breakfast with 100 cc. of water.	10-11	0 97	265	19 4	12 4	13 1	24
12 noon lunch with 1000 cc. of water.	11-12	0 90	285	18 9	12 9	13 9	25
1:40 p.m., 500 cc. of water. Cutaneous blood	12-1	2 17	135	22 1	12 5*		17*
	1-2	5 08	57	20 5	13 2*		18*
	2-3	6 37	50	19 6	15 2*		20*
Exp. No. 14-a. Val.	10-11	1 87	222	14 9*	14 9*		21*
9 a.m., 100 cc. of water. Venous blood	11-12	0 88	361	26 1	11 5	12 6	23

Exp. No. 19. Val. 7:30 a.m., dry breakfast. 1 p.m., dry lunch. Cutaneous blood	9-10	1 02	0 96	419	. 29 6	13 6	13 9	26			
	10-11	0 90	0 85	429	29 2	12 4	13 5	25			
	11-12	0 70	0 66	473	29 5	10 6	13 0	24			
	12-1	0 70	0 66	492	30 2	10 8	13 2	24			
	1-2	0 87	0 82	417	29 1	11 7	13 0	24			
	2-3	0 80	0 75	418	28 5	11 1	12 7	24			
	9-10	0 83	0 78	499	32 9	11 9	13 4	25			
	10-11	0 97	0 91	466	30 8	13 8	14 4	27			
	11-12	1 00	0 94	469	31 3	14 1	14 5	27			
	12-1	1 53	1 44	321	30 2	15 3*		20*			
	1-2	3 42	3 21	131	29 3	14 4*		19*			
	2-3	1 27	1 19	306	29 8	12 2*		16*			
	9-10	0 78	0 73	520	32 1	11 9	17 7	33			
Exp. No. 21. Val. 7:30 a.m., dry breakfast 12:05 p.m., lunch with 1000 cc. of water. Cutane- ous blood	10-11	0 83	0 78	504	30 8	12 9	14 4	27			
	11-12	0 77	0 72	495	30 5	11 7	13 8	26			
	12-1	1 70	1 60	285	30 6	14 9*		20*			
	1-2	4 08	3 83	121	30 2	15 3*		20*			
	2-3	2 20	2 07	203	30 0	13 9*		19*			
	11-1	2 29	2 17	234	50 2	10 2*		14*			
	Exp. A-19 Gia. Admitted to hospital 10:30 a.m. Venous blood	9-11	1 54	1 46	247	46 2	7 9	6 47	12		
		9-11	0 75	0 71	304	38 2	5 7	6 72	12		
		9-11	0 90	0 85	305	34 0	7 6	8 29	15		
		9-11	0 93	0 88	319	39 9	7 1	7 52	14		
		Exp. A-20. Gia. Fasting. Venous blood	9-11	0 75	0 71	304	38 2	5 7	6 72	12	
			9-11	0 90	0 85	305	34 0	7 6	8 29	15	
			9-11	0 93	0 88	319	39 9	7 1	7 52	14	
Exp. A-21. Gia. 8:15 a.m., 100 cc. of water. Venous blood			9-11	0 75	0 71	304	38 2	5 7	6 72	12	
			9-11	0 90	0 85	305	34 0	7 6	8 29	15	
			9-11	0 93	0 88	319	39 9	7 1	7 52	14	
			Exp. A-22. Gia. 8:25 a.m., 100 cc. of water. Venous blood	9-11	0 75	0 71	304	38 2	5 7	6 72	12
				9-11	0 90	0 85	305	34 0	7 6	8 29	15
				9-11	0 93	0 88	319	39 9	7 1	7 52	14
	Exp. A-23. Gia. 8:20 a.m., 100 cc. of water. Venous blood			9-11	0 75	0 71	304	38 2	5 7	6 72	12
				9-11	0 90	0 85	305	34 0	7 6	8 29	15
				9-11	0 93	0 88	319	39 9	7 1	7 52	14

TABLE 3—Continued

Time	V Urine volume	V cor. Urine volume corrected for body size by factor 1.73 Area	U Urine urea nitrogen	B Blood urea nitrogen	$\frac{UV \text{ cor.}}{B}$ Observed clearance*	$\frac{U\sqrt{V} \text{ cor.}}{B}$ Calculated stand- ard and below clearance (for V) augmentation limit	Per cent of average normal clearance
Exp. A-24. Gia. 8:20 a.m., 100 cc. of water. Venous blood	9-11 1.02	0.97	269	34.8	7.4	7.61	14
Exp. A-25. Gia. 8:20 a.m., 100 cc. of water. Venous blood	9-11 0.80	0.76	314	35.3	6.8	7.76	14
Exp. A-26. Gia. 9:05 a.m., 100 cc. of water. Venous blood	9-11 1.04	0.99	339	37.0	9.0	9.10	17
Exp. A-27. Gia. 8:25 a.m., 100 cc. of water. Venous blood	9-11 0.97	0.92	388	44.6	8.1	8.36	15
Exp. A-28. Gia. 8:30 a.m., 100 cc. of water. Venous blood	9-11 0.97	0.92	362	39.4	8.4	8.82	16
Exp. A-29. Gia. 6 a.m., 30 grams urea and 500 cc. of water. 7, 8, 9, 10, and 11 a.m., 500 cc. of water each time. Venous blood	9-10 2.17 10-11 3.37 11-12 4.17	2.06 3.20 3.96	362 268 209	71.9 70.4 69.0	10.3* 12.2* 12.1*		14* 16* 16*
Exp. A-30. Gia. 8:25 a.m., 100 cc. of water. Venous blood	9-11 1.17	1.11	435	49.9	9.7	9.19	17

Exp. No. 24. Wol. 7:30 a.m., breakfast with 1000 cc. of water. 9:30 a.m., 500 cc. of water. 12:05 p.m., lunch with 1000 cc. of water. Cutaneous blood	9-10 10-11 11-12 12-1 1-2 2-3	2 33 1 98 1 53 1 37 2 93 2 63	2 82 2 40 1 85 1 66 3 55 3 18	141 142 147 141 123 126	27 8 27 4 27 0 27 9 27 5 26 2	14 3 12 5  8 5 16 0 15 4	8 52 8 14 7 42 6 52 8 42 8 58	16 15 14 12 16 16
Exp. No. 24-a. Wol. 9 a.m., 100 cc. of water. Venous blood	10-11 11-12	1 17 0 67	1 42 0 81	383 425	45 2	11 9 7 6	10 10 8 47	19 16
Exp. No. 27. Wol. 7:30 a.m., breakfast with 1000 cc. of water. Cutaneous blood	9-10 10-11 11-12	2 38 1 77 0 80	2 88 2 14 0 97	126 122 43	25 5 23 2 24 2	14 3 11 3 6 3	8 39 7 69 6 23	16 14 12
Exp. No. 30. Wol. 8:30 a.m., breakfast with 1000 cc. of water. 9:50 a.m., 250 cc. and 11:20 a.m., 500 cc. of water. 11:50 a.m., lunch with 500 cc. of water. 1:10 p.m., 300 cc. of water. Cutaneous blood	9-10 10-11 11-12 12-1 1-2 2-3 10-11	2 12 1 93 1 43 1 67 1 97 1 97 0 75	2 57 2 33 1 73 2 02 2 38 2 38 0 91	110 109 118 109 103 93 164	22 5 21 9 24 6 21 0 22 3 23 1 21 4	12 5 11 7 8 2 10 5 10 9 9 7 7 0	7 83 7 61 6 32 7 39 7 13 6 22 7 30	14 14 12 14 13 12 14
Exp. No. 30-a. Wol. 9 a.m., 100 cc. of water. Cutaneous blood	9-10	2 08	2 52	151	37 4	10 0	6 40	12
Exp. No. 30-b. Wol. 6 a.m., 300 cc. of water and 15 grams urea. 7, 8, 9, 10, and 11 a.m., 300 cc. of water each time. Cutaneous blood	10-11 11-12	2 08 2 47	2 52 2 99	147 134	35 9 35 2	10 3 11 3	6 49 6 58	12 12

\* Clearance figures marked \* represent maximum clearance values determined when  $V$  was above the augmentation limit of the subject. For augmentation limits see table 4.



been calculated as in the preceding paper. The results are given in table 3. For the sixth patient (Wol.) such a calculation was not possible because of his failing ability to put out large volumes of water.

The points determined after previous ingestion of urea are indicated on the charts by black discs, those determined on the days when no urea had been given, by hollow circles. It is apparent from figures 2 and 3 that the results are not to any important degree influenced by urea ingestion.<sup>1</sup>

Two facts are at once apparent from the figures. The first is that the urea excretion curves of these patients in five cases out of six are much lower than any observed normal curve. The second is that the pathological curves resemble the normal ones in that, when urea excretion is plotted against the square root of urine volume, each curve is composed of 2 straight lines, an ascending line showing urea excretion increasing with urine volume over the lower ranges of the latter, and a horizontal line, at higher volume ranges, representing maximum excretion. These two lines intersect, as in normal subjects, at an augmentation limit. In the case of patient Wol., only the sloping first part of the curve remains, diminution of power to excrete water had rendered unattainable the urine volumes represented on all or nearly all of the horizontal part. In patient Val., on the other hand, most of the ascending part is lost through loss of power of concentration. Only enough remains to suggest the bend of the curve at the augmentation limit.

<sup>1</sup> Addis (Arch. Int. Med., 1922, xxx, 378) considers it essential to place the kidneys under strain by administration of urea and water in order to force them to maximum effort, whereby functional deficits should be revealed that would otherwise escape notice. The idea is logical, and is supported by Addis' results with partially nephrectomized animals. We have accordingly expected to find some patients in the initial stages of declining renal function, who would still show normal standard and maximum clearances under ordinary living conditions, but would reveal deficits in the maximum clearance when fed urea and water under the conditions of Addis' test. This expectation has, however, never been realized. In observations on patients with the various types of Bright's disease, with all variations in renal function from normal to practically zero, we have not yet encountered one in whom the conditions of the Addis test revealed a functional deficit when the blood urea clearance, determined without urea feeding, did not do so, nor have we seen any in whom the degree of deficit found was significantly increased by imposing the conditions of the Addis test.

In figure 7 the same urea excretion curve as the one given in figure 5 has been plotted on logarithmic paper. In the logarithmic curve variations in height are proportional to *percentage* changes, rather than absolute changes, in the data plotted. In a uremic case, the clearance values are all so low that variations on them are inconspicuous, when plotted on an ordinary scale, as in figure 5. But when plotted logarithmically, as in figure 7, they are as evident as the clearance variations of a normal subject.

The values for the augmentation limits, given in table 4, are on the whole somewhat lower than those found for normal subjects in the preceding paper (6). The average figures for nephritic and normal subjects are 1.73 and 2.13 cc. per minute respectively. The decrease in augmentation limit is hardly great enough to justify the conclusion that it represents an effect of the disease. It is not very significant compared with the relatively great fall in the *level* of the curves observed in cases with damaged renal function.

In each case there is, compared with the normal, a fall of nearly equal proportions in the level of the ascending line and in that of the horizontal line reached at the augmentation limits, with relatively small change in the limit. Consequently the standard blood urea clearance, indicated by the height of the ascending line at  $V = 1$  cc. per minute, and the maximum clearance, indicated by the height at the augmentation limit and beyond, show in these cases approximately equal percentage diminutions below the normal. The similarity in the significance of results by the maximum and standard clearance determinations is also indicated by the agreement between the percentages of normal values shown by the two clearances for each individual, indicated by the figures with and without stars, respectively in the last column of table 3.

In table 4 the variations for the standard and maximum clearances in each patient are given. The table shows that, as previously found with normal individuals, the average variation in a given subject is slightly greater for the standard clearance than for the maximum clearance.

In figure 8 all the curves are presented, with scales indicating the per cent of normal standard and maximum clearance observed in each case. It is apparent that both clearances tend to show about the same percentage fall in cases with renal deficiency.



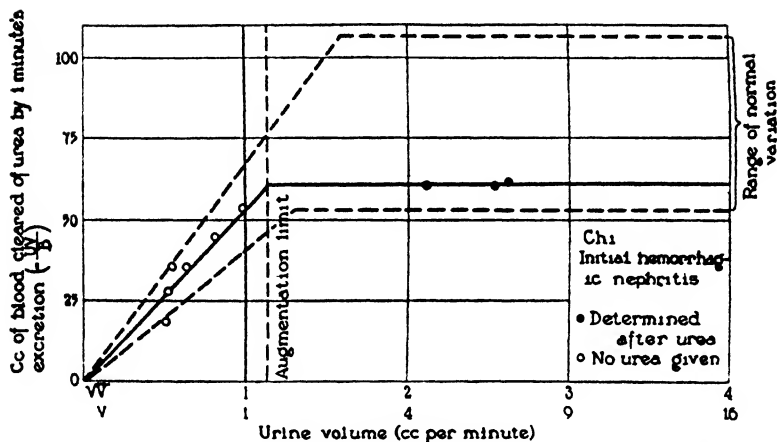


FIG. 1. BLOOD UREA CLEARANCE CURVE, CORRECTED FOR BODY SIZE, OF PATIENT CHI.

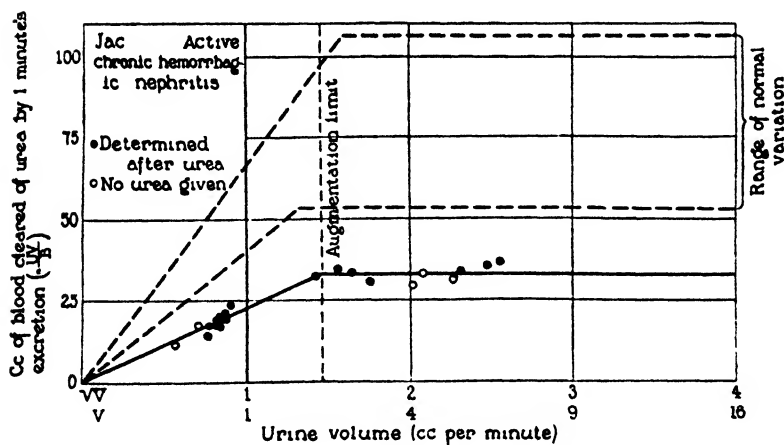
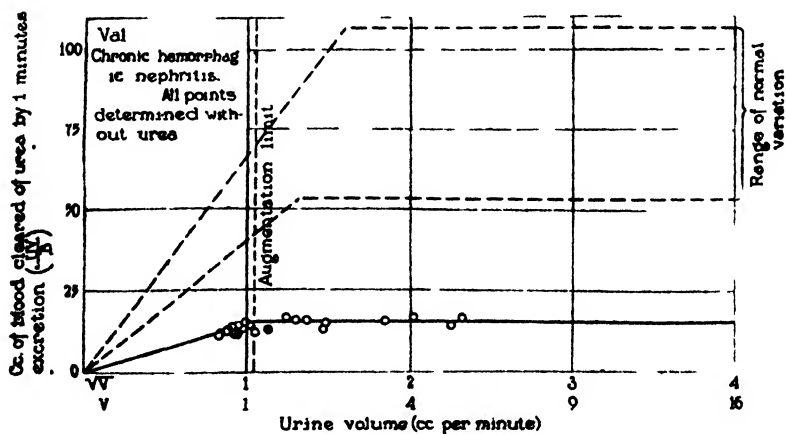
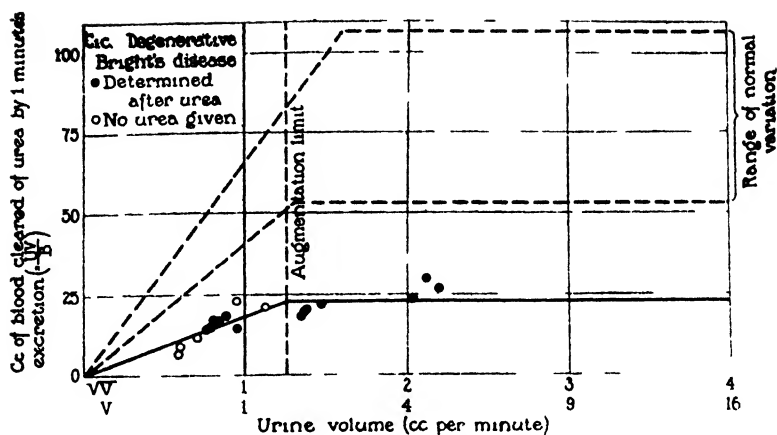


FIG. 2. BLOOD UREA CLEARANCE CURVE, CORRECTED FOR BODY SIZE, OF PATIENT JAC.



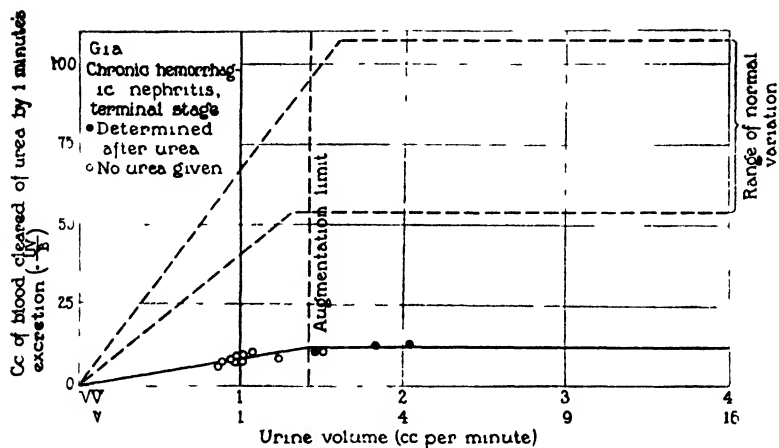


FIG. 5. BLOOD UREA CLEARANCE CURVE, CORRECTED FOR BODY SIZE, OF PATIENT GIA.

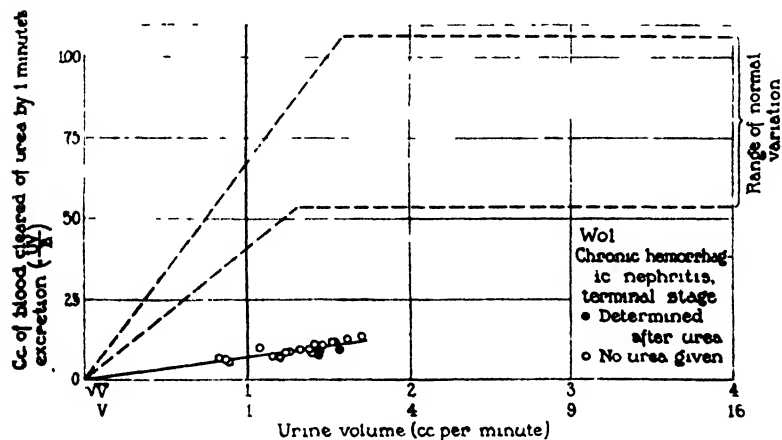


FIG. 6. BLOOD UREA CLEARANCE CURVE, CORRECTED FOR BODY SIZE, OF PATIENT WOL.

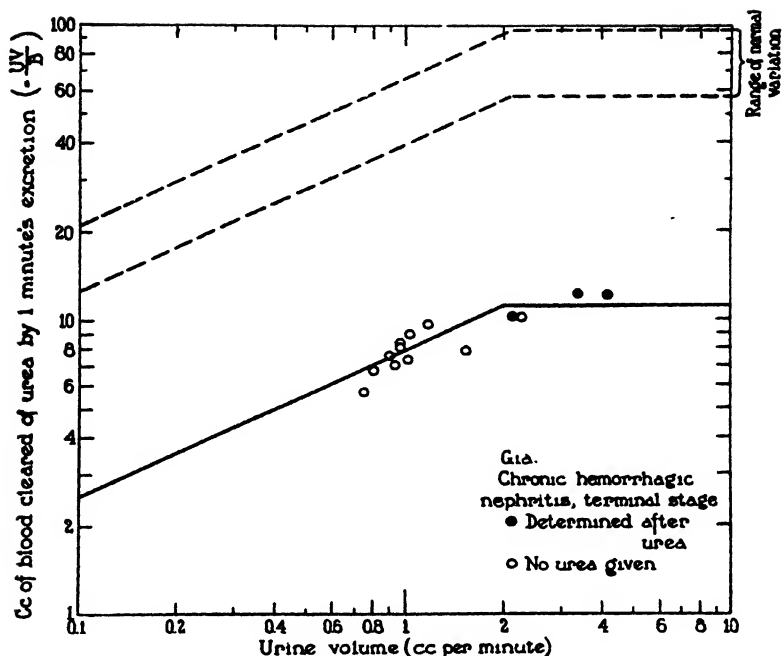


FIG. 7. THE SAME UREA CLEARANCE CURVE AS THE ONE GIVEN IN FIGURE 5 BUT HERE PLOTTED ON LOGARITHMIC PAPER

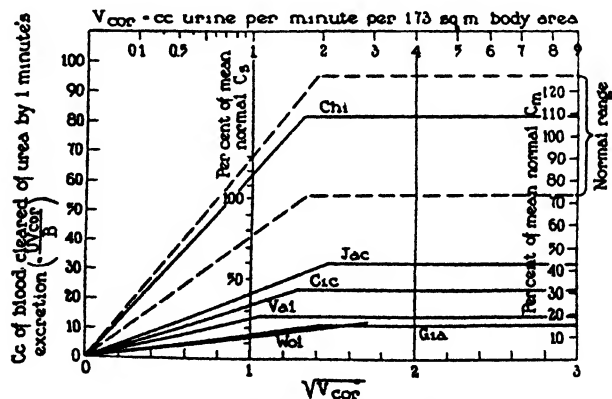


FIG. 8. CURVES OF THE SIX PATIENTS, SHOWING RELATIVE EFFECTS OF RENAL DEFICIENCY OF EACH ON STANDARD AND MAXIMUM CLEARANCES

Of the 5 cases with urea excreting power, as indicated by the blood urea clearances, consistently reduced to from 40 to 12 per cent of the average normal, all except one (Gia.), showed at times blood urea nitrogen below the maximum (23 mgm. per cent) found by MacKay and MacKay (4) in normal subjects. These results confirm the conclusion of these authors (3), that more than half the functioning tissue of the kidneys may be destroyed before the blood urea rises above normal limits.

In subjects with such renal loss the blood urea may, in fact, even be less than the normal average. Thus in experiments 10-a and 20-c blood urea nitrogen of only 10 to 12 mgm. per 100 cc. is seen, despite the fact that both subjects showed only about 40 per cent of mean normal excreting power. Such results indicate the uncertainty attending interpretation of normal blood urea values in nephritic patients if the urea excretion rate is not also taken into consideration. It has in fact been common on our wards to find consistently normal blood ureas in nephritic patients who have lost 40 to 60 per cent of their renal function. Such patients are likely, either by choice or direction, to take diets low in protein: and if they consume half as much as a given normal subject, other factors being equal, they will show about normal blood urea content. If, in addition, they drink more water, they may have even less blood urea than many normal subjects. In the cases to be presented in a later paper there are numerous examples of this fact.

#### SUMMARY

1. Data are presented showing the relationship between urine volume and urea excretion in 6 patients with Bright's disease, in stages varying from an acute case with normal urea excreting ability to an advanced chronic case with only one-eighth of normal urea excreting power.

2. The rate of urea excretion in these patients was found to vary with blood urea content and urine volume in the same manner as in normal subjects.

3. The standard blood urea clearance, representing the cubic centimeters of blood cleared of urea by 1 minute's excretion when urine volume is 1 cc. per minute, and the maximum clearance, with

urine volumes above the augmentation limit of about 2 cc. per minute, proved to be equally sensitive as indicators of renal function in these nephritics.

4. Our data confirm MacKay and MacKay (3) in showing that loss of renal function may exceed 60 per cent before the blood urea content rises above the highest level observed in normal subjects. Unless the excretion rate is also considered, the blood urea, taken alone, may fail to reveal diminishing renal ability until the latter has reached an advanced stage.

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## STUDIES OF UREA EXCRETION.

### V. THE DIURNAL VARIATION OF UREA EXCRETION IN NORMAL INDIVIDUALS AND PATIENTS WITH BRIGHT'S DISEASE.

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In the preceding papers (4, 5, 6) the applicability of the relationship  $C_s = \frac{U}{B} \sqrt{V_c}$ , as a measure of the urea excreting ability of the kidneys under conditions of low or normal urine volumes, has been demonstrated. This relationship has been called the *standard blood urea clearance* or *standard clearance*, and represents the number of cubic centimeters of blood which are cleared of urea when the urine volume is at the average normal level of 1 cc. per minute. The relative constancy of this measure in a given normal or nephritic individual or in a group of normal individuals shows that the blood urea content and the urine volume are the two factors which appear to be ordinarily of chief importance in regulating the urea output. However, the fact that in a given individual the probable variation of the standard clearance is  $\pm 10$  per cent and that the maximum variation is much greater indicates that other factors in addition to the blood urea concentration and urine volume affect urea excretion. At high urine volumes, where the rate is uninfluenced by the volume, the effects of some of these factors have been studied (1). Before pursuing a study of the effect of specific factors on the rate of urea excretion at low urine volumes as measured by the *standard clearance* the fluctuation of this value with the ordinary activities of the day and night have been observed. The diurnal variation of the *standard clearance* has been followed in several normal individuals and in a number of patients suffering from Bright's disease.

## METHODS

In each experiment observations were made over a 24-hour period, from 6 a.m. one morning until 6 a.m. on the next day. Hourly urine specimens were collected from 6 a.m. until 10 p.m., and then a single specimen was collected between 10 p.m. and 6 a.m. When the subject was unable to void or there was doubt concerning the completeness of voiding the hour period was extended to 2 hours. A sample of blood was drawn by vein puncture at 6:30 a.m., the middle of the first urine collection period, and then at the middle of each second hour thereafter until 8:30 p.m. The blood urea values for the intermediate hours were obtained by interpolation. A sample was drawn at 9:30 p.m. for the last urine period of the day, and another at 6 a.m. on the following morning. The average value of these two samples served as the blood urea concentration from which the standard clearance of the night period was calculated. Urine collections were made within 2 minutes, and the blood samples were drawn within 5 minutes of the stated time. The blood and urine urea concentrations were determined gasometrically (7). The standard clearance,

$C_s = \frac{U}{B} \sqrt{V_c}$ , where  $U$  is the urine urea concentration,  $B$  the blood

urea concentration, and  $V$  the urine volume in cubic centimeters per minute, was calculated as previously described (4, 5). The urine volume, is corrected in each case to  $V_c$  by the use of a factor dependent on the ideal body surface of the subject. On the charts the standard clearance has been recorded as the actual value and as a per cent of the normal mean of 54 cc. per minute. When  $V_c$  was above the augmentation limit of 2 the rate of urea excretion has been calculated on the

basis of the maximum blood urea clearance,  $\frac{U V_c}{B}$  (5). These are

recorded in the figures as a per cent of the mean normal, 75 cc. per minute, but have not been used in determining the variability of the rate of urea excretion.

The normal subjects on whom observations were made were up and about during the course of the experiments. All of the patients suffering from Bright's disease were confined to bed. None of the latter received any coffee with their meals, while the normal subjects



had coffee with their breakfast. There were no dietary restrictions in either case although most of the patients happened to be receiving a diet which contained very little sodium chloride.

#### NORMAL SUBJECTS

In figure 1 have been charted the results obtained on four normal individuals. Although there are many differences between the four

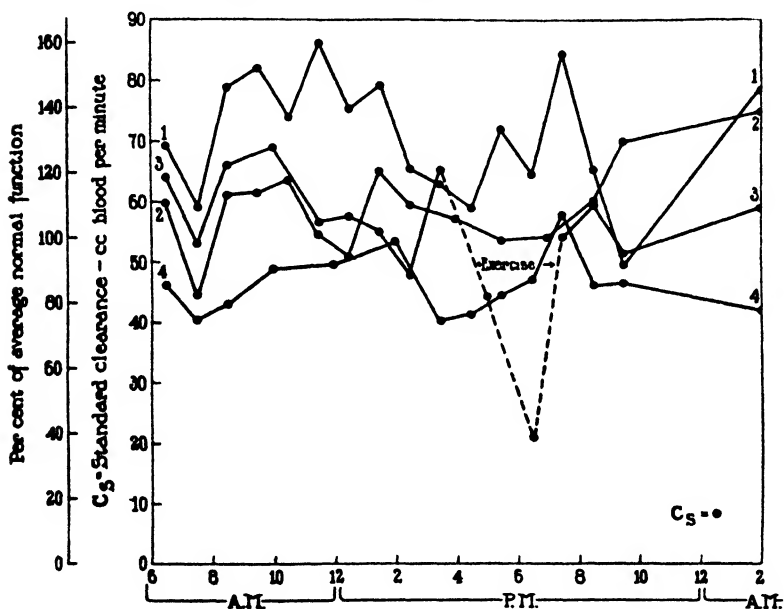


FIG. 1. NORMAL SUBJECTS

curves there are common points characteristic of all of them. In every case the standard clearance is depressed during the first hour after arising. Following this, but commencing before breakfast, there is a regular increase. In a general way this higher level continues through the morning. It is during this period, from 9 to 12 a.m., that the standard clearance shows the least variation, an important consideration in its practical application as a test of renal function, when choosing the time of day during which figures are to be obtained.

After the lunch hour there is a definite drop in the clearance values, which then rise again during the late afternoon and evening. There is no certain effect of meals. In all 4 cases there is a decrease in the clearance an hour after lunch and in 3 cases a similar fall in its value after dinner. The standard clearance for the period of sleep was determined from the actual urine volume and urine urea concentration by the use of the average of the evening and morning blood urea concentrations. The relation of the figures obtained during the night to the daytime values is not constant.

One of the most interesting findings is the observations on subject 3 during the late afternoon, a period in which he indulged in several sets of tennis. A marked depression of his standard clearance occurred. Exercise has been shown (1) to cause likewise a reduction in the maximum blood urea clearance. In both cases this probably follows a decrease in the rate of renal blood flow resulting from the increased blood supply demanded by the muscular tissues. The varying blood supply requirement of different organs and tissues resulting from digestion and other physiological activity, by its influence upon the amount of blood flowing through the kidneys, may well be the cause of much of the variation in the value of the standard clearance. The larger the value of the standard clearance the greater is the variability (table 2) in this figure.

#### PATIENTS WITH BRIGHT'S DISEASE

Observations of the standard clearance were made each hour during the day on 15 patients suffering from different forms of Bright's disease (figs. 2, 3, and 4). In their grouping Addis' classification (2) has been used. The clinical and laboratory data which form the basis of their separation comprise table 1. The classification of the formed elements of the urine is according to Addis (2).

The two patients with hemorrhagic Bright's disease who had a nearly renal normal function (nos. 5 and 6) showed the same drop in their clearance during the first hour after awakening that was displayed by the normals. In every case the clearance values tend to increase during the course of the day, and they all fall during the period of sleep. The variability of the clearance values decreases in general with the level of the clearance (table 2). Subject 11 was nearly

comatose, and inaccurate voidings probably account for the exceptional variability with such a low function. In percentages of the mean observed clearance values, the variability of the observations on patients is greater than of those obtained on normal subjects. This may be partly due to the fact that the former were all confined to bed where it is more difficult to empty the bladder completely than in the standing posture. Incomplete urine collections could not, however,

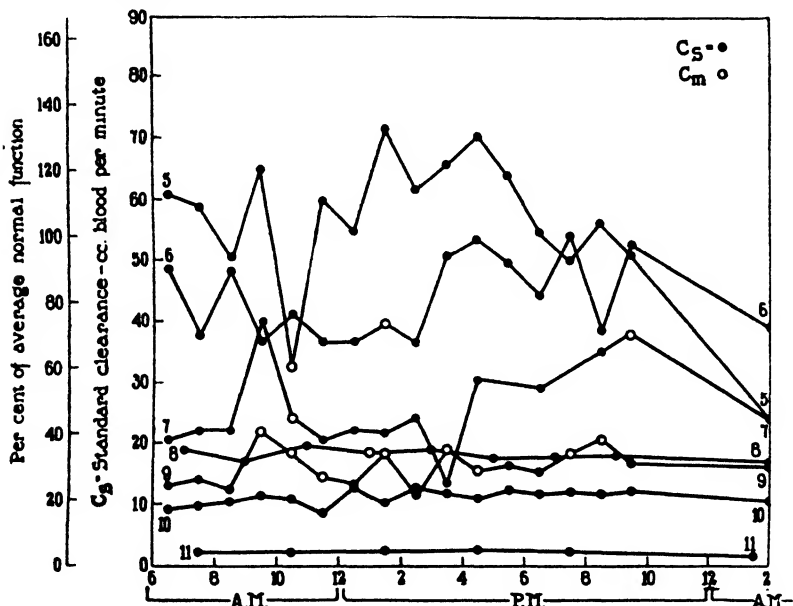


FIG. 2. HEMORRHAGIC BRIGHT'S DISEASE

account for all of the difference. As in the case of the normals the least variable standard clearances occur in every case between the hours of 9 and 12 a.m.<sup>1</sup>

<sup>1</sup> Some of the observations recorded in figs. 2, 3, and 4 were made when the urine volumes exceeded the augmentation limit, and accordingly yielded maximum instead of standard clearances. The  $C_m$  values, represented as hollow circles, are given in percentages of the mean normal  $C_m$  of 75 cc. blood per minute. The outer scale at the left of each figure applies both to  $C_5$  and  $C_m$  values, the inner scale only to  $C_5$ .

Observations on 5 patients with degenerative Bright's disease are given in figure 3. With the exception of case no. 12, one on whom

TAB  
*Clinical and laborat*

Case number	Hospital number	Diagnosis		Complications	Age	Sex	Heart size	Blood pressure	Eye grounds	Edema
		Bright's disease								
		Type	Stage							
5	6458	Hemorrhagic	Initial—latent	Acute sinusitis	49	M	Normal	155/106	Normal	0
6	6164	Hemorrhagic	Healed		31	M	Normal	125/65	Normal	0
7	6475	Hemorrhagic	Active		25	F	Normal	168/114	Normal	++
8	6139	Hemorrhagic	Latent		19	M	Normal	152/94	Normal	0
9	6162	Hemorrhagic	Latent		20	F	Slightly increased	202/115	Normal	0
10	6238	Hemorrhagic	Initial—terminal	Cardiac insufficiency	16	M	Increased	136/86	Normal	0
11	6166	Hemorrhagic	Terminal		34	F	Increased	203/118	Retinitis with hemorrhages	+
12	6184	Degenerative cryptic	Active		18	M	Normal	106/77	Normal	+++
13	6473	Degenerative cryptic	Initial		12	M	Normal	115/70	Normal	+++
14	6172	Degenerative cryptic	Active		Pulmonary tuberculosis	29	M	Normal	110/68	Normal
15	5949	Degenerative cryptic	Terminal	Empyema	20	M	Normal	128/83	Normal	+++
16	5505	Degenerative cryptic	Terminal	Cardiac failure	11	M	Normal	116/60	Normal	++
17	6446	Arteriosclerotic			49	F	Normal	234/154	Normal	0
18	6102	Arteriosclerotic			51	F	Slightly increased	148/86	Normal	0
19	6466	Arteriosclerotic			49	F	Greatly increased	242/145	Retinitis	+
20	5210	Hemorrhagic	Terminal		Otitis media	30	M	Normal	148/190	Normal
21	5482	Hemorrhagic	Active		12	M	Normal	152/110	Normal	+

highly variable figures have always been obtained, the results are essentially similar. Except for one case they all show the fall in the clearance in the morning after awakening. The increase in the clear-

ance during the course of the day, which was evident in all of the normals and the patients with hemorrhagic Bright's disease, is absent

LE 1  
ory data on patients

Blood									Urine (excretion per 12 hours)														Renal function	
Urea nitrogen	N P N	Plasma		Plasma proteins					Protein	Formed elements											Phthalen excretion (2 hours)	Standard urea clearance		
		Creatinine	Cholesterol	Hemoglobin	Albumin	Globulin	Total	A/G ratio		Erythrocytes	Leucocytes and epothelial cells	Casts												
												Total	Hyaline	Blood	Fatty	Epithelial	Granular	Waxy	Failure					
mgm per cent	mgm per cent	mgm per cent	mgm per cent	ml mms per cent O <sub>2</sub>	per cent	per cent	per cent		gms	mil lions	mil lions	mil lions	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc per min ute				
10.6	35.0	1.30	500	18.5	3.54	3.13	6.67	1.13	1.8	19.15	11.74	0.31	100	0	0	0	0	0	66.9	5.8				
12.7	29.0	1.42	250	19.2	4.52	2.16	6.68	2.10	0.0	1.01	0.11	0.01	100	0	0	0	0	0	60.6	43.9				
8.8	27.0	1.36		15.0	1.23	2.68	3.91	0.46	2.9	25.46	26.80	12.46	90	0	8	0	2	0	56.4	24.9				
23.8	48.0	1.97		16.1	4.32	2.54	6.86	1.70	1.6	13.49	11.48	1.08	83	3	0	0	14	0	37.0	18.1				
12.5	28.0	1.58	457	17.0	3.06	2.08	5.14	1.47	2.5	11.02	3.39	1.75	100	0	0	0	0	0	41.0	14.3				
46.1	64.0	4.39	279	11.5	3.07	2.54	5.61	1.21	4.6	190.68	77.20	4.48	67	13	0	0	12	0	14.1	11.7				
100.0	259.0	16.00	314	10.9	3.4	2.72	6.19	1.27	2.5	8.19	8.77	0.76	0	0	0	0	100	0	1.0	2.2				
6.6	20.0	1.33	539	10.1	1.34	3.47	4.81	0.39	6.5	0.31	6.82	1.86	70	0	20	10	0	0	6.7	44.8				
18.8	28.0	1.36	600	13.0	1.65	2.32	3.97	0.71	3.8	0.06	14.11	0.88	65	0	35	0	0	0	50.1	24.7				
12.7	33.0	1.67	839	17.1	1.92	2.72	4.64	0.70	7.5	0.33	24.48	5.30	80	0	16	4	0	0	54.6	18.1				
37.3	50.0	3.10	682	10.5	1.96	2.90	4.86	0.68	3.7	0.39	38.53	7.57	10	0	10	10	25	5	7.0	16.6				
22.3	49.0	3.16	648	19.9	1.29	3.12	4.41	0.41	3.9	0.40	4.29	1.48	30	0	0	0	43	0	10.2	12.0				
11.4	14.0	1.36	313	20.0	4.46	2.81	7.27	1.59	0.3	0.58	0.32	0.32	100	0	0	0	0	0	74.0	47.7				
15.7	36.0	1.67		19.0	4.32	3.05	7.37	1.42	0.1	0.58	0.43	0.14	100	0	0	0	0	0	50.0	42.2				
31.1	57.0	1.76		15.7	3.21	2.79	6.00	1.15	0.8	3.04	0.23	0.08	50	0	0	0	50	0	28.9	6.0				
46.2	61.0	4.50	288	12.1	2.35	2.22	4.57	1.06	3.2	21.12	4.93	0.67	35	17	13	26	0	0	16.0	8.0				
12.1	22.0	1.50		16.7	1.70	2.79	4.49	0.61	5.6	7.05	4.70	6.30	90	0	10	0	0	0	35.7	16.0				

in this group, and in two cases quite the reverse occurs. Here again the variability decreases with the decrease in the standard clearance (table 2). In case no. 17, who suffered from enuresis, the variability

ance during the course of the day, which was evident in all of the normals and the patients with hemorrhagic Bright's disease, is absent

.E 1

ry data on patients

Blood										Urine (excretion per 12 hours)													Renal function	
No.	N P N.	Plasma		Plasma proteins				A/G ratio	Protein	Formed elements										Phthalain excretion (2 hours)	Standard urea clearance			
		Creatinine	Cholesterol	Hemoglobin	Albumin	Globulin	Total			Erythrocytes	Leucocytes and epithelial cells	Casts												
												Total	Hyaline	Blood	Fatty	Epithelial	Granular	Waxy	Failure					
mgm per cent	mgm per cent	mgm per cent	mgm per cent	vol- umes per cent O <sub>2</sub>	per cent	per cent	per cent	gms	mil- lions	mil- lions	mil- lions	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	cc per minute				
6	35 0	1 30	500	18 5	3 54	3 13	6 67	1 13	1 8	19 15	11 74	0 31	100	0	0	0	0	0	66 9	55 8				
7	29 0	1 42	250	19 2	4 52	2 16	6 68	2 10	0 0	1 01	0 11	0 01	100	0	0	0	0	0	60 6	43 9				
8	27 0	1 36		15 0	1 23	2 68	3 91	0 46	2 9	25 46	26 80	12 46	90	0	8	0	2	0	56 4	24 9				
8	48 0	1 97		16 1	4 32	2 54	6 86	1 70	1 6	13 49	11 48	1 08	83	3	0	0	14	0	37 0	18 1				
5	28 0	1 58	457	17 0	3 06	2 08	5 14	1 47	2 5	11 02	3 39	1 75	100	0	0	0	0	0	41 0	14 3				
1	64 0	4 39	279	11 5	3 07	2 54	5 61	1 21	4 6	190 68	77 20	4 48	67	13	0	0	12	0	8	14 1	11 1			
0	259 0	16 00	344	10 9	3 4	2 72	6 19	1 27	2 5	8 19	8 77	0 76	0	0	0	0	100	0	100	1 0	2 2			
6	20 0	1 33	539	10 1	1 34	3 47	4 81	0 39	6 5	0 31	6 82	1 86	70	0	20	10	0	0	67 8	44 8				
8	28 0	1 36	600	11 0	1 65	2 32	3 97	0 71	3 8	0 06	14 11	0 88	65	0	35	0	0	0	50 1	24 7				
7	33 0	1 67	839	17 1	1 92	2 72	4 64	0 70	7 5	0 33	24 48	5 30	80	0	16	4	0	0	54 6	18 1				
3	30 0	3 10	682	10 5	1 96	2 90	4 86	0 68	3 7	0 39	38 53	7 57	10	0	10	10	25	5	40	7 0	16 6			
3	49 0	3 16	648	19 9	1 29	3 12	4 41	0 41	3 9	0 40	4 29	1 48	30	0	0	0	43	0	27	10 2	12 0			
4	14 0	1 36	313	20 0	4 46	2 81	7 27	1 59	0 3	0 58	0 32	0 32	100	0	0	0	0	0	74 0	47 1				
7	36 0	1 67		19 0	4 32	3 05	7 37	1 42	0 1	0 58	0 43	0 14	100	0	0	0	0	0	50 0	42 2				
1	57 0	1 76		15 7	3 21	2 79	6 00	1 15	0 8	3 04	0 23	0 08	50	0	0	0	50	0	0	28 9	6 0			
2	61 0	4 50	288	12 1	2 35	2 22	4 57	1 06	3 2	21 12	4 93	0 67	35	17	13	26	0	0	9	16 0	8 0			
1	22 0	1 50		16 7	1 70	2 79	4 49	0 61	5 6	7 05	4 70	6 30	90	0	10	0	0	0	35 7	16 0				

in this group, and in two cases quite the reverse occurs. Here again the variability decreases with the decrease in the standard clearance (table 2). In case no. 17, who suffered from enuresis, the variability

is rather high due to our inability to obtain accurate specimens. In 4 of the 5 subjects the standard clearance fell during the period of sleep.

TABLE 2  
*Values for standard clearance*

Case number	Number of observations	C <sub>s</sub> average	Variability*
Normal subjects			
		<i>cc per minute</i>	<i>per cent</i>
1	17	71.3	17.9
2	15	59.4	12.9
3	13	58.2	10.5
4	14	46.2	10.2
Hemorrhagic Bright's disease			
5	17	55.8	20.3
6	16	43.9	15.0
7	13	24.9	28.7
8	8	18.1	4.9
9	9	14.3	12.5
10	17	11.2	10.6
11	6	2.2	12.1
Degenerative Bright's disease			
12	15	44.5	30.5
13	17	24.7	24.2
14	17	18.1	22.9
15	17	16.6	11.7
16	10	12.0	20.4
Arteriosclerotic Bright's disease			
17	15	47.7	38.0
18	14	42.2	32.3
19	15	6.0	28.3

\* The average variation of the individual observations from the mean value for each series expressed as a per cent of the mean.

Three patients with arteriosclerotic Bright's disease were examined (fig. 4). The characteristics of the other curves are entirely missing here, and the variability of the observations (table 2) is by far the greatest of any of the groups. Subject 17 showed the largest fluctua-

tions. This patient had a rather unstable vaso-motor system and a variable but constantly high arterial blood pressure. The second patient (no. 18) was a phlegmatic individual with only a slight hypertension. The third subject (no. 19) had a very low function produced in part by a complicating cardiac insufficiency. The low variability associated with a low standard clearance in the other groups is lacking here. Whether a high variability of the standard clearance values is

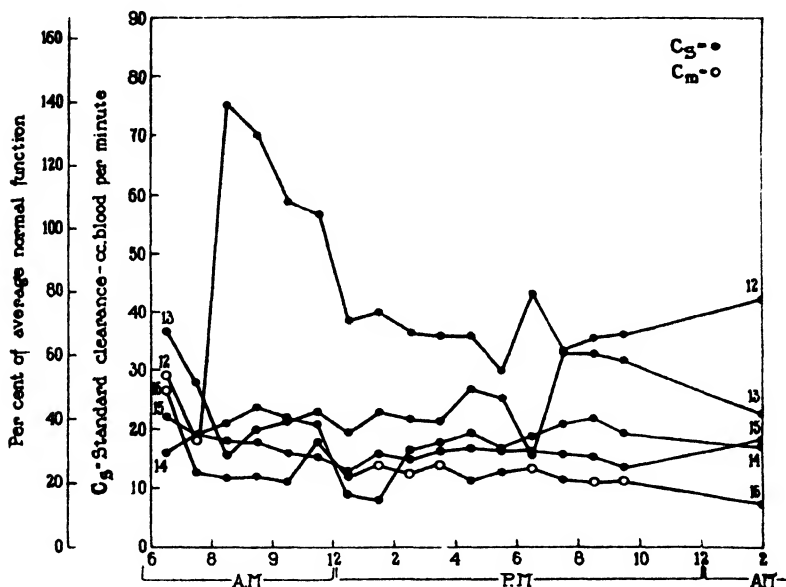


FIG. 3. DEGENERATIVE BRIGHT'S DISEASE

always associated with this type of renal lesion is not certain. That it should be the case is not surprising for the clearance figures are probably largely dependent on the state of the renal vascular system, and both the superficial and visceral blood vessels of individuals with hypertension are known (3) to be hypersensitive to normal stimuli.

#### THE EFFECT OF MEALS

In nearly every normal subject or patient, on whom observations of the standard clearance during the day have been made, the least



variable values occur between the hours of 9 and 12 a.m. It is during this period that observations for clinical use have always been made on patients in our wards. This period follows the breakfast hour, and from the point of view of the practical use of the standard clearance as a measure of renal function it becomes important to determine whether or not meals, especially breakfast, have any effect upon the clearance figures. In the normal individuals who were examined

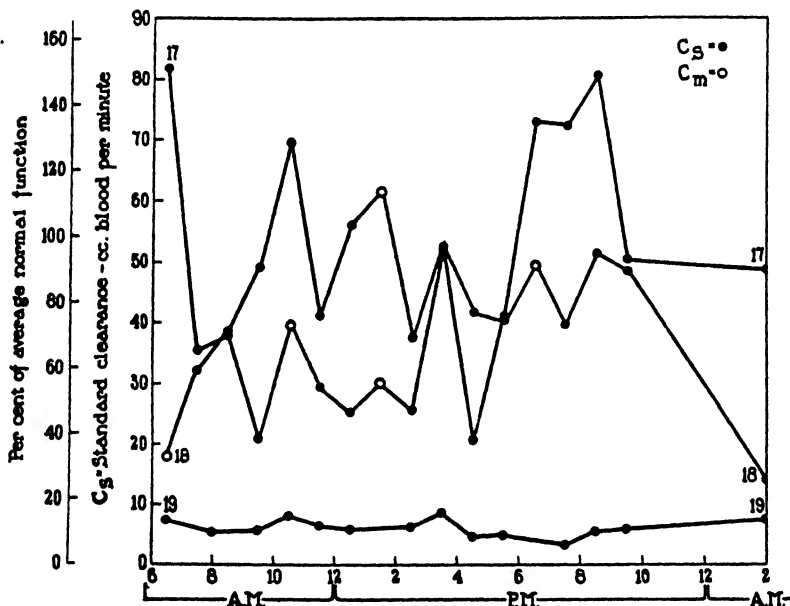


FIG. 4. ARTERIOSCLEROTIC BRIGHT'S DISEASE

there appeared to be a decrease in the value of the clearance after both breakfast and lunch. From the observations on patients no conclusion can be reached on this point.

In order to ascertain whether it is necessary to carry out this urea excretion test in the fasting state a series of observations were made on two patients with hemorrhagic Bright's disease. Observations were made for two hours, from 6 to 8 a.m. before breakfast, and for two other hour periods, 9 to 11 a.m. after breakfast. The tests were

**TABLE 3**  
*Testing effect of breakfast on standard clearance*

Case number	Breakfast protein content	C <sub>s</sub>		Effect
		Before breakfast	After breakfast	
21	<i>grams</i>			<i>per cent</i>
	11	10 4	11 0	+5 8
	15	9 9	10 4	+5 1
	24	9 1	8 2	-9 9
	35	8 4	8 3	-1 9
	41	7 3	7 8	+6 9
	35	7 1	9 2	+29 6
	27	7 9	7 5	-5 1
	18	6 6	6 6	0 0
	10	5 6	6 7	+19 6
Average.		8 0	8 4	+5 1
22	19	15 2	14 6	-3 9
	23	16 5	16 5	0 0
	28	12 8	12 3	-3 9
	34	18 6	20 7	+11 3
	21	13 0	12 7	-2 3
	23	16 5	18 1	+9 7
	15	19 1	21 2	+11 0
Average.		16 0	16 6	+3 1

**TABLE 4**  
*Testing effect of breakfast on standard clearance*

Case number	Number of observations	C <sub>s</sub>	
		Breakfast given	Breakfast omitted
2	1	<i>cc per minute</i> 64 0	<i>cc per minute</i> —
	2	—	64 5
	3	62 6*	—
	4	—	58 3
	5	56 4	—
	6	—	57 4
Average.		61 0	60 1
14	1	18 5	—
	2	—	18 7
	3	22 4	—
	4	—	20 5
	5	19 6*	—
	6	—	21 0
Average. ..		20 2	20 1

\* Coffee taken.

made four days apart. No coffee was given. The results are given in table 3. Breakfast has no effect, for the volume of blood cleared of urea per minute was in each subject consistently the same before and after the meal. The average figures show a slight increase in the post-breakfast figures, but it is not significant. It is accordingly unnecessary to limit measurements of the standard clearance to fasting periods. As additional proof that breakfast has no demonstrable effect, daily observations of the standard clearance were made between 8 and 10 a.m. on a normal subject (no. 2), and a patient (no. 14) with degenerative Bright's disease. On alternate days breakfast was omitted. The results in table 4 show that there was no effect.

The experiments detailed in table 3 indicate that there is less variability in a series of observations made on an individual on different days, but at the same time each day, than in a series of observations all made on the same day.

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## VIRUS III ENCEPHALITIS.

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PLATES 12 TO 14.

(Received for publication, July 6, 1928.)

In 1923 Rivers and Tillett (1) described reactions in rabbits which were considered to be induced by the virus of varicella. Subsequently, however, in Rivers' (2, 3) laboratory and also in Swift's (4, 5), it was found that the reactions were caused not by the virus of varicella but by an unknown virus indigenous to rabbits which had been accidentally encountered in the work on chicken-pox and rheumatic fever. Inasmuch as the spontaneous disease caused by the virus has not been recognized, no name other than Virus III has as yet been given this active agent. The term is used merely for convenience and designates the third strain of the virus encountered with which most of the work happens to have been conducted.

The character of Virus III lesions and the presence of acidophilic nuclear inclusions (3) in the injured tissues led Rivers and Tillett to do cross-immunity experiments to determine what relationship, if any, Virus III bears to herpetic virus (2). None was found. Furthermore, in 1924, rabbits were inoculated intracerebrally with Virus III to determine if it was capable of producing an encephalitis (2). A temperature above 104°F., which persisted for a week and which was much more marked in the experimental than in the control animals, was the only abnormality noted. Because of the mild reaction no further study of Virus III encephalitis was made at that time.

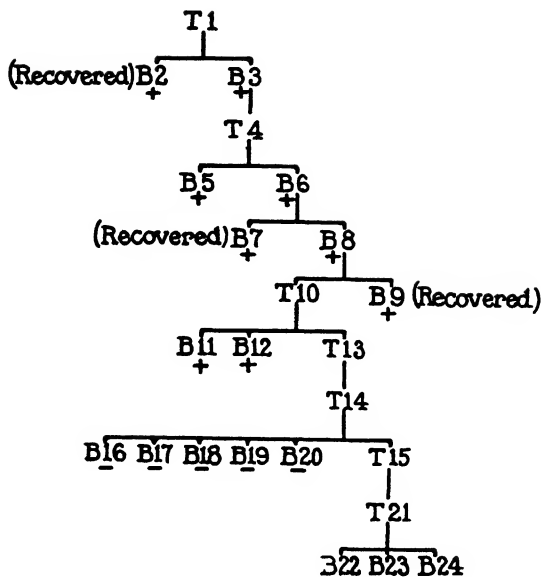
From the time of discovery of Virus III, early in 1923, until September, 1926, when emulsions of tissues containing the virus were frozen, desiccated, sealed in tubes, and stored on ice, testicular passages (approximately 300) were made at intervals of 3 or 4 days. In January, 1928, the dried virus was removed from the ice box and testicular passages were resumed. In the course of some experiments,

intracerebral inoculations with the virus were made in rabbits and signs of encephalitis, which were followed by death in a number of instances, were observed. It is with this encephalitis caused by Virus III that the present paper deals.

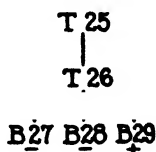
#### EXPERIMENTAL.

*Methods and Materials.*—In sealed tubes on ice, frozen and desiccated Virus III (6) retains its activity indefinitely. Its activity is also maintained for at least 6 weeks if infected testicular emulsions are mixed with equal amounts of glycerol, sealed, and stored on ice. Experiments, however, were always conducted with fresh material. Either emulsions of infected testicles or brain emulsions containing the virus served this purpose. The emulsions were prepared by grinding the infected tissues with sand in a mortar and then adding enough Locke's solution to make a 20 per cent suspension. To free the material from sand, centrifugation at low speed for 1 minute was employed. 2,000 gm. rabbits were used. 0.2 cc. of an emulsion was the amount chosen for intracerebral or for intradermal inoculation, and 1.0 cc. for intratesticular inoculation. Even though it does not so appear in the text-figures, at least two rabbits were inoculated each time the virus was passed. In working with Virus III this is necessary because an immune animal is occasionally encountered which results in the loss of the virus. The sterility of all tissues was tested by means of aerobic and anaerobic cultures. All operations were performed under ether anesthesia. Tissues for histological studies were fixed in Zenker's fluid and stained either with eosin-methylene blue or by Giemsa's method.

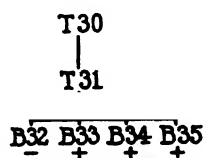
In Text-figs. 1 to 3 are outlined the methods of procedure employed in the study of Virus III encephalitis. Although the majority of intracerebral inoculations was made with testicular virus, it will be seen in Text-fig. 1 that the virus propagated itself through three successive intracerebral passages, causing in each instance definite signs of encephalitis, *e.g.*, tremor, ataxia, irritability, circling, salivation, retention of urine, generalized tonic and clonic contractions of the skeletal muscles, or paralysis. From the text-figures it will also be observed that potent testicular virus did not produce signs of encephalitis in every rabbit of certain series and that the results of intracerebral inoculations varied considerably in the different series of animals, *i.e.*, at times all the rabbits died, while on other occasions none showed signs other than a pathological increase in temperature. There is no adequate explanation for this remarkable variation in activity of the virus when inoculated intracerebrally.



TEXT-FIG. 1. Outline of procedure employed in the study of Virus III encephalitis. *T* indicates site of inoculation (testicle) and also organ emulsion (testicle) used for next passage of virus. *B* indicates site of inoculation (brain) and also organ emulsion (brain) used for next passage of virus. + indicates occurrence of definite clinical signs of encephalitis. - indicates absence of clinical signs of encephalitis other than fever. Rabbit T 1 was inoculated intratesticularly with glycerolated Virus III 5 passages removed from an animal inoculated with the desiccated material that had been stored on ice more than a year.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 2. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 25 was inoculated intratesticularly with glycerolated Virus III from Rabbit T'1.

TEXT-FIG. 3. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 30 was inoculated intratesticularly with desiccated Virus III that had been stored on ice more than a year.

*Rabbit B 5.*

March 6. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 4.

March 7. Temperature 104.2°; animal appears normal. March 8. Temperature 105.0°; animal appears normal. March 9. Temperature 103.4°; sick and irritable. Occasional generalized tonic and clonic contractions of the skeletal muscles. March 10. Temperature 101.0°; paralysis of hind legs; retention of urine. March 11. Paralysis; retention of urine; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous; vessels distended with blood. No evidence of a purulent meningitis. Brain fixed in Zenker's fluid. Sections stained with eosin-methylene blue and according to Giemsa's method.

*Section through Hippocampal Region.*—Slight general thickening of pia-arachnoid with cellular infiltration consisting of many endothelial leucocytes, a few lymphocytes, and rare polymorphonuclear cells. Occasional acidophilic nuclear inclusion (Fig. 7) in endothelial leucocytes. In places, fixed endothelial cells are prominent and rarely contain inclusions; few mitotic figures; slight perivascular infiltration of penetrating vessels. Generalized meningeal hyperemia with a moderate amount of hemorrhage. Few nuclear inclusions in cells that probably are arachnoidal fibroblasts. Hemorrhage in wall of third ventricle, accompanied by very slight reactive changes. Nuclear inclusions in cells of ependyma (Fig. 6) and choroid plexus. In foci in hippocampus are numerous typical nuclear inclusions; the cellular degeneration is associated with very little, if any, reaction.

*Section through Cerebellum.*—Meningeal lesions similar to those described above; marked cortical hemorrhage. Nuclear inclusions in outer cells of molecular layer. Pycnotic and fragmentary degeneration of nerve cells of granular layer (Fig. 5). Marked hyaline necrosis of Purkinje cells with pycnosis and chromatolysis of nuclei (Fig. 1). No inclusions found in Purkinje cells.

*Rabbit B 6.*

March 6. Inoculation similar to that of Rabbit B 5. March 7. Temperature 104°; animal appears normal. March 8. Temperature 105°; animal appears normal. March 9. Temperature 102°; sick and irritable. March 10. Paralysis of hind legs. March 11. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. March 12. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous. Aerobic and anaerobic cultures showed no growth. Part of brain used for passage of virus, remainder fixed in Zenker's fluid for histological study.

*Section through Hippocampal Region.*—Diffuse meningitis; some edema, hemorrhage, and fibrin. Nearly all of the cells are endothelial leucocytes; occasional typical nuclear inclusion. Nothing of importance observed in hippocampus.

*Section through Cerebellum.*—Meningeal reaction similar to that described above.

Some diffuse and marked focal degeneration of Purkinje cells as indicated by the striking oxyphilic reaction involving both the nucleus and the cytoplasm; chromatolysis, karyorrhexis, and a fading out of the Purkinje cells together with adjacent cells of the molecular layer. Throughout the degenerating areas the lack of a significant degree of inflammatory response is noteworthy.

### *Rabbit B 7.*

March 12. Inoculated intracerebrally with 0.2 cc. of fresh brain Virus III from Rabbit B 6.

March 13. Temperature 102.8°; animal appears normal. March 14. Temperature 103.4°; animal appears normal. March 15. Temperature 104.0°; sick. March 16. Temperature 104.2°; sick. March 17. Temperature 104.5°; sick. March 18. Sick. March 19. Temperature 101.2°; sick; head pulled to left and rotated. March 20. Temperature 100.5°; ataxia; loss of weight; head pulled to left; circling. March 21. Temperature 100.0°; retention of urine. March 22. Animal prostrate. March 23. Began to improve and eventually recovered.

### *Rabbit B 8.*

March 12. Inoculation similar to that of Rabbit B 7.

March 13. Temperature 102.8°; animal appears normal. March 14. Temperature 102.4°; animal appears normal. March 15. Temperature 105.8°; sick; irritable; head retracted. March 16. Temperature 104.0°; condition worse. March 17. Temperature 105.0°; condition same. Animal sacrificed for passage of the virus and for histological studies. Cultures of the brain showed no growth.

*Sections through the Point of Inoculation and also through the Hippocampus.*—Diffuse meningitis—the cells chiefly endothelial leucocytes; a slight generalized invasion of peripheral cortical tissue both over the surface of the brain and also beneath the ependyma. Some perivascular thickening due to endothelial cells. Numerous characteristic nuclear inclusions in endothelial leucocytes and some in arachnoidal fibroblasts, especially in those applied along large vessels. Hyaline degeneration of scattered nerve cells in periphery of cortex. About the site of inoculation, hemorrhage, spongy degeneration of the parenchyma, and a confused cellular picture are observed. All cells are swollen; nuclei are reduced to peripheral chromatin rings with central inclusions. No other structure is sufficiently preserved to enable absolute identification of specific cell type, but it appears that all structures contain inclusions—endothelial, nerve, and glia cells. Marked degeneration of hippocampal cells; swollen nuclei; numerous typical nuclear inclusions.



*Rabbit B 29.*

April 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 26.

April 20. Temperature 104.8°; animal wild. April 21. Temperature 104.0°; animal wild. April 22. Temperature 105.4°; animal wild. April 23. Temperature 104.5°; tremor. April 24. Temperature 103.0°; marked tremor and ataxia; circling to left; head pulled to left; salivation; twitching of muscles around mouth and of fore legs; occasional generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed for histological studies. Brain perfused with Zenker's fluid.

The usual meningitis with more polymorphonuclear cells than usually seen. Isolated Purkinje cells show typical hyaline necrosis and nuclear chromatolysis. Nuclear inclusions in ependymal cells lining fourth ventricle and in neighboring glia cells.

Very severe degenerative changes in hippocampus (Fig. 4) with nuclear inclusions in almost every cell (Fig. 2) and extensive spongy degeneration in fiber layers. Slight perivascular hemorrhage. Perivascular infiltration (Fig. 8) consisting of polymorphonuclear cells, lymphocytes, and endothelial leucocytes.

Spongy degeneration and necrosis of nerve cells with typical nuclear inclusion in thalamic region. Epithelial cells of choroid plexus contain nuclear inclusions.

Meningitis with some of the cells showing nuclear inclusions extends to the cervical cord. Slight involvement of periphery of cord and of perivascular sheaths.

*Rabbit B 35.*

May 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 31.

May 20. Temperature 102.8°; animal seems normal. May 21. Temperature 104.9°; animal seems normal. May 22. Temperature 104.0°; sick. May 23. Temperature 104.0°; worse; tremor. Tremor persisted until May 26, when the rabbit was sacrificed. The brain was perfused with Zenker's fluid. This animal showed no signs of encephalitis except fever, tremor, and a tendency to stand rigidly in one position for long periods of time.

Meninges show lymphocytic, endothelial and plasma cell infiltration, fibrin, and hemorrhage. Nuclear inclusions in endothelial leucocytes and fixed endothelium. Perivascular lymphocytic infiltration along penetrating vessels.

Hyaline necrosis with nuclear karyorrhexis of many Purkinje cells. Nuclear inclusions in small nerve cells interspersed between Purkinje cells, in glia cells, and in endothelial leucocytes. Spongy degeneration of associated fiber layer.

Extensive spongy degeneration with nuclear inclusions in nearly every cell in the hippocampus. Some inclusions in peripheral cortical glia cells and in invading endothelial leucocytes.

A few typical inclusions in glia cells in periphery of cervical cord. Slight endothelial cell infiltration.

In addition to Virus III encephalitis this brain also showed lesions of the spontaneous encephalitis described by Wright and Craighead (7).

From the results of the experiments described above it is obvious that the active agent used is capable of producing an encephalitis in rabbits. The question naturally arises, however, as to whether the virus now under investigation is the one originally encountered 5 years ago, or whether it has become contaminated by another virus, *e.g.*, vaccine virus or herpetic virus.

*Relation of Present Virus III to Original Virus III.*

Repeated experimental passages of the virus in animals that are occasionally spontaneously infected make it impossible to say definitely that the original strain of Virus III has not been contaminated by a new strain of the active agent. A fortunate circumstance, however, enabled us to demonstrate that the present Virus III is identical with, or at least similar to, the original Virus III. In 1925 Rivers and Pearce (8) found that the transplantable rabbit neoplasm described by Pearce and Brown (9) is infected with Virus III and that the virus persists in the tumor and is regularly passed from rabbit to rabbit with each successive transfer of the tumor. In view of these facts, in order to establish a relationship between the present Virus III and the Virus III of 1925 it was only necessary to determine whether the tumor rabbits are refractory to the active agent now being used. For this purpose Dr. Pearce supplied 6 rabbits that had shown good growths of the tumor.

6 tumor rabbits were inoculated intradermally and intracerebrally respectively with 0.2 cc. of a fresh testicular emulsion containing virus of the same generation as Rabbit B 6 (Text-fig. 1). As controls, an animal which had recovered from encephalitis caused by our virus was inoculated in a similar manner, and 2 normal stock animals received intradermal and intratesticular inoculations. The results showed that the recovered animal and the 6 tumor rabbits had no reaction in the skin and evidenced no signs of encephalitis, while the normal animals had a very marked reaction in the skin and a high fever.

From the results of the above experiment one is justified in concluding that in all probability the virus now being used is identical with the original Virus III.

*Consideration of Possible Contaminants.*

The next question to arise dealt with the possibility that the emulsions containing Virus III were contaminated by the virus of vaccinia, rabies, or herpes.

*Vaccine Virus.*—One can be quite positive that the emulsions containing Virus III do not also contain vaccine virus, inasmuch as Dr. Pearce's tumor rabbits, not immune to vaccine virus, are completely protected against the activity of our emulsions. Furthermore, no Guarnieri bodies were observed in cells injured by our active agent.

*Rabic Virus.*—In making repeated passages in rabbits, it is not inconceivable that one might rarely encounter the virus of rabies. This possibility is very remote. Moreover, Dr. Pearce's tumor animals are immune to our virus, even when inoculated intracerebrally. If rabic virus were a contaminant, her animals, although immune to Virus III, would die of rabies. This did not occur, nor were Negri bodies found in the brains of rabbits dying of Virus III encephalitis.

*Herpetic Virus.*—The clinical and pathological picture presented by our animals at times so closely resembles that caused by herpetic virus that one naturally would like to know whether our emulsions are contaminated by the virus of herpes.

5 rabbits were chosen; 1 was a normal stock animal, the other 4 had recovered, 4 to 8 weeks previously, from Virus III encephalitis. Each animal was inoculated intracerebrally with 0.2 cc. of a brain emulsion containing H. F. herpetic virus. All of the rabbits showed the usual signs of herpetic encephalitis and were dead within 7 days.

The above experiment clearly indicates that our virus is not contaminated by herpetic virus.

## DISCUSSION.

Virus III is an active, filterable agent indigenous to rabbits (2, 5). It undoubtedly causes a natural infection in these animals, yet the spontaneous disease has not as yet been recognized. As previously shown (1-3), the virus under experimental conditions produces a high fever and characteristic lesions in the cornea, testicles, and skin. Furthermore, within epithelial and endothelial cells of these lesions

acidophilic nuclear inclusions, similar to those seen in varicella and herpes, occur (3).

The studies described in the present paper clearly indicate that Virus III at times is capable of inducing in rabbits an encephalitis which clinically and pathologically closely resembles that caused by herpetic virus. The most interesting fact disclosed by the present work, however, is that the ability of the virus to produce visible evidences of encephalitis seems to vary greatly from time to time (Text-figs. 1 to 3). No adequate explanation of this striking feature is now available. The question as to whether the frequent passages of the virus under experimental conditions, with an occasional freezing and desiccation or storage in glycerol, have altered its activity cannot be answered at present.

The histopathology of experimental Virus III meningoencephalitis in rabbits resembles in part that of herpetic encephalitis. The two diseases are pathologically similar in that both produce a "chronic" type of meningitis, characterized by lymphocytic, plasma cell, and endothelial cell infiltration (Fig. 7); the perivascular sheaths of penetrating vessels may be distended by similar cells (Fig. 8). In both diseases the hippocampal region is profoundly involved; nerve cells, glia cells, and endothelial leucocytes contain characteristic acidophilic nuclear inclusions (Fig. 2); nerve cells undergo hyaline degeneration and seem to disappear rapidly, leaving a spongy, reticulated zone of ground substance (Figs. 2, 4). The adjacent fiber laminae likewise present a soft, spongy appearance and a few polymorphonuclear and endothelial leucocytes infiltrate the region.

In Virus III encephalitis the next most prominent lesion occurs in the Purkinje cell layer of the cerebellum (Figs. 1, 5). These large cells undergo hyaline necrosis accompanied by nuclear chromatolysis, pycnosis, and karyorrhexis; no inclusions have been observed in their nuclei. Inclusions, however, frequently occur in the smaller nerve cells, in glia cells, and in reacting endothelial leucocytes in the immediate vicinity. The necrosis and disappearance of Purkinje cells leave a zone of spongy degeneration between the granular and molecular layers of the cerebellum. In some animals extensive pycnotic degeneration of nerve cells in the granular layer of the cerebellum (Fig. 5) also occurs. Some of the peculiar clinical manifestations of

the disease are probably due to the cerebellar lesions, but in view of multiple foci of brain involvement one should be cautious in relating the clinical picture to lesions in different anatomical foci.

Meningeal edema and hemorrhage, small hemorrhages at the site of inoculation, and foci of hemorrhage in the deep pontine region further complicate the picture. In some rabbits superficial cortical and subependymal encephalitis was noted; here one finds a spongy degeneration, mitosis of glia and endothelial cells, and infiltration by endothelial leucocytes.

It seems that no type of cell escapes involvement. Inclusions have been seen in nerve cells, glia cells, fixed and mobile endothelial cells, arachnoidal fibroblasts, ependymal cells, and in cells of the choroid plexus. Often one cannot distinguish between a glia cell and an infiltrating endothelial leucocyte because of the loss of characteristic nuclear structure; involved nuclei are reduced to a central, often elongated, inclusion surrounded by a clear zone limited externally by a narrow ring of altered chromatin.

The presence of inclusions, the disappearance of cells and ground substance giving rise to the peculiar soft, spongy, reticular appearance, and the minimal amount of inflammatory reaction agree well with the findings in encephalitis caused by other filterable viruses.

#### SUMMARY.

Virus III, an active, filterable agent indigenous to rabbits, under experimental conditions produces, in addition to lesions in the cornea, skin, and testicles, an encephalitis which is at times quite similar to that induced by herpetic virus. Virus III and herpetic virus, however, are not immunologically related.

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## EXPLANATION OF PLATES.

## PLATE 12.

FIG. 1. Cerebellum. Hyaline necrosis of Purkinje cells. Zenker; eosin-methylene blue.  $\times 500$ .

FIG. 2. Hippocampus. Cellular degeneration; abundant inclusion bodies in nuclei. Zenker; eosin-methylene blue.  $\times 1000$ .

FIG. 3. Site of inoculation. Large granular nuclear inclusion in nerve cell. Zenker; eosin-methylene blue.  $\times 1500$ .

## PLATE 13.

FIG. 4. Necrosis of hippocampal cells; spongy degeneration of fiber layers; hyperemia and slight cellular reaction. Zenker; eosin-methylene blue.  $\times 115$ .

FIG. 5. Pycnotic degeneration of nerve cells of granular layer of cerebellum. Zenker; eosin-methylene blue.  $\times 210$ .

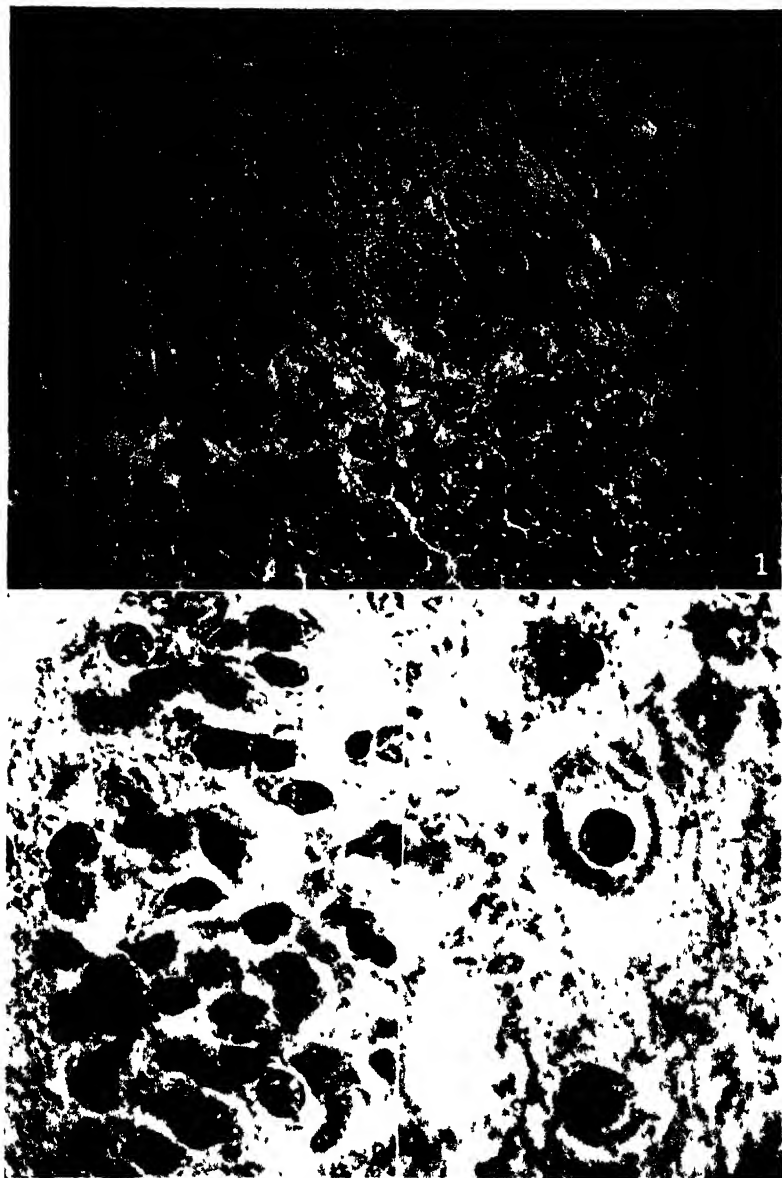
## PLATE 14.

FIG. 6. Inclusions in ependymal cells lining third ventricle. Zenker; eosin-methylene blue.  $\times 1000$ .

FIG. 7. Endothelial reaction in cortical meninges. Three nuclear inclusions, *a, b, c*. Zenker; Giemsa.  $\times 850$ .

FIG. 8. Cortex showing perivascular infiltration. Zenker; eosin-methylene blue.  $\times 130$ .

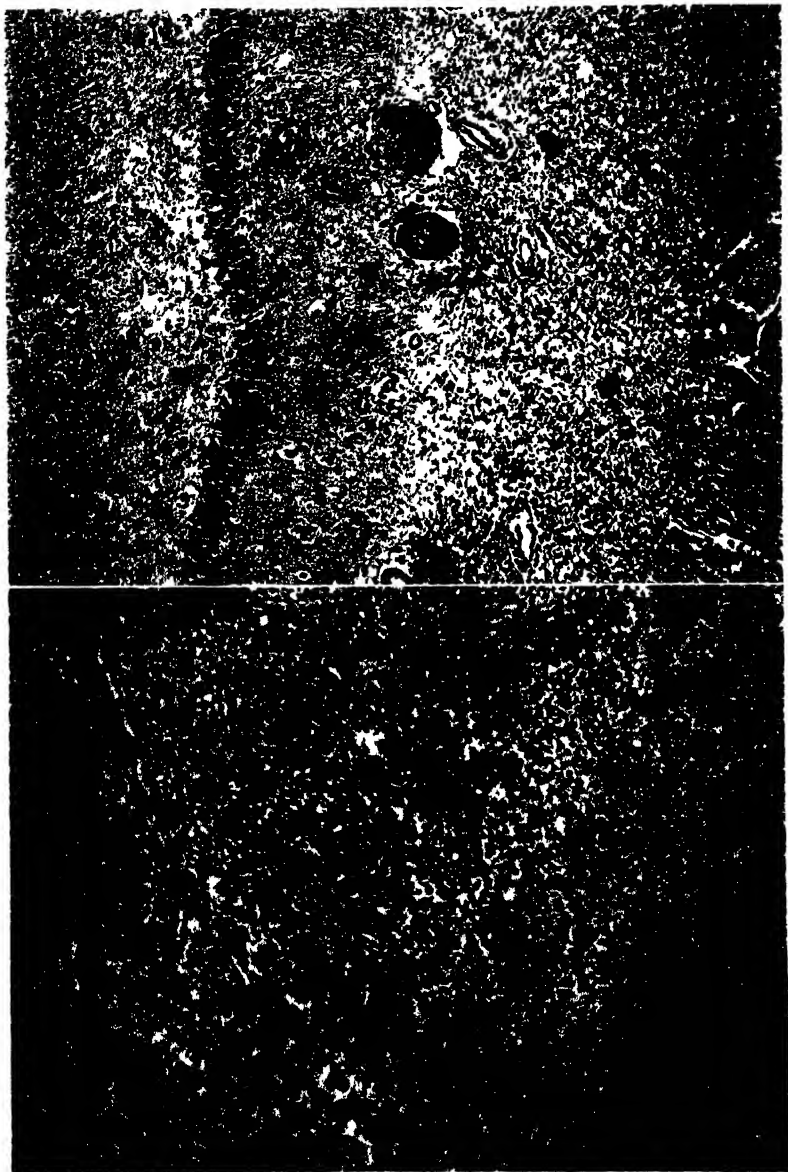




Photographed by Louis Schmidt







Photographed by Louis Schmidt

(Rivers and Stewart Virus III encephalitis)





Photographed by Louis Schmidt

(Rivers and Stewart Virus III encephalitis)



## STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

### V. THE INCIDENCE OF CARRIERS DURING THE ENDEMIC STAGE.

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(Received for publication, July 5, 1928.)

The course of two *Salmonella* infections appearing spontaneously in a guinea pig population has been discussed in previous papers (1, 2). As noted earlier, the guinea pigs comprising the particular population are divided into two groups, breeders and weaned young or stock. Animals employed in experimental work are withdrawn from the latter group. From time to time *B. paratyphi* has been isolated from the spleen of stock guinea pigs injected with other bacteria. It seemed probable that the guinea pigs were carrying *B. paratyphi* in the spleen as a result of earlier exposure to infection.

A routine bacteriological examination of all guinea pigs that died had been carried out for several years. Positive cultures were rarely obtained from the spleen with unweaned guinea pigs in the absence of gross lesions. However, no definite study of carriers within the population at large had been attempted. Consequently a comprehensive examination of breeders and young guinea pigs, just prior to weaning, was undertaken. In addition a new breeding group composed of selected sows and boars was brought together and isolated from the main population. The selected group was kept under observation for evidence of reinfection.

Before considering the work on carriers a more detailed statement concerning the extent of the two infections may be pertinent. The specific percentage mortality for the population (including the selected breeders) during that year is given in Table I. The usual monthly fluctuations occurred. The rates for the second infection, introduced into the population a year earlier, were in general higher than those for the first form.

When the examination for carriers was begun the breeding stock was composed of some 209 animals. These were maintained in a separate animal unit, distributed in large metal cages containing 4 or 5 sows and 1 boar. The group was a heterogeneous one comprising animals from 1 to 5 years of age. A few of the number had passed through the active stage of the initial outbreak of paratyphoid in 1924. The majority of breeders, however, were younger animals added shortly before or at intervals after the appearance of the second infection during the summer of 1926. Generally the individuals in any one cage were of approximately the same age.

Cages containing a full quota of guinea pigs were selected and brought to the laboratory for examination. It may be said that pregnant sows are removed to

TABLE' I.

*Population, Total Deaths, Deaths from Paratyphoid, and Percentage Mortality from Paratyphoid from September, 1927, through June, 1928.*

Month	Popula- tion	Total deaths	Deaths from Para- typhoid Type I	Mortality Type I	Deaths from Para- typhoid Type II	Mortality Type II
				<i>per cent</i>		<i>per cent</i>
September.....	513	11	1	0.19	6	1.16
October.....	561	17	3	0.53	11	1.96
November.....	668	23	1	0.14	5	0.74
December.....	573	27	2	0.34	3	0.52
January.....	661	60	3	0.45	13	1.96
February.....	631	39	2	0.31	6	0.95
March.....	595	62	0	0.00	1	0.16
April.....	497	73	1	0.20	1	0.20
May.....	493	43	2	0.40	1	0.20
June.....	483	75	1	0.20	3	0.62

individual units just prior to parturition. In all, 105 animals were transferred, representing 20 cages. The entire work extended over a period of 5 months. Two successive fecal cultures were made from each guinea pig and the agglutinin content of the blood serum determined for the two types of *B. paratyphi*.

### *General Methods.*

The following routine procedures were carried out.

The guinea pigs were first bled. In most instances sufficient blood was obtained from incising an ear vein after shaving and the application of xylol. 13 of the total number were refractory and heart puncture under ether was resorted to, with no fatalities. Serum from the coagulated samples was used in

testing. The antigens were fresh, standardized, saline suspensions of the two *Salmonella* types. The final dilution series in a total volume of 1 cc. ranged from 1:20 through 1:1280. The tubes were incubated at 37°C. for 3 hours and were read after overnight refrigeration. In Tables III and IV the results are expressed in terms of the limiting dilution, the highest dilution showing any macroscopic evidence of agglutination.

After bleeding, the guinea pigs were transferred to separate cages with clean bedding. The next morning a fecal sample was collected. The bedding was changed and on the following morning a second sample collected. The fresh feces, at least two pellets, were emulsified in 5 cc. of plain broth and incubated for 5 to 6 hours at 37°C. Two large loops were then transferred to a tube of malachite green-lead acetate broth.

Difficulty had been experienced with this medium in former work. Hydrogen sulfide in amounts detectable by lead acetate was produced only when fermented bouillon was employed. The peptone used was apparently deficient in the particular sulfur compound utilized for the production of hydrogen sulfide. With the fermented bouillon some available sulfur compound was evidently produced during growth of the fermenting bacteria. It may be noted that *B. coli* is added to the meat infusion and allowed to grow at incubator temperature. After steaming and filtering, peptone is added as usual. Tilley (3) recommended the addition of sodium thiosulfate to media employed for hydrogen sulfide production. This compound was substituted for fermented broth with excellent results. The medium as finally employed was beef infusion broth containing 1 per cent peptone, of an initial pH of 7.6, and tubed in 5 cc. amounts. Immediately before use there was added 0.05 cc. of a 5 per cent aqueous solution of sodium thiosulfate, 0.25 cc. of a 1 per cent solution of lead acetate, and 0.15 cc. of a 0.2 per cent aqueous malachite green solution. The addition of the lead acetate causes a bulky precipitate which settles rapidly. In this medium *B. paratyphi* and other members of the *Salmonella* group produce a uniformly turbid growth. The precipitate blackens during the first 24 hours and there is a distinct odor of hydrogen sulfide. Motility is retained but is less active than in plain bouillon. Other intestinal bacteria are generally restrained through 48 hours at 37°C. *B. proteus*, however, may develop with the production of hydrogen sulfide. Occasionally there is growth of unidentified bacteria with no change in color of the precipitate.

The tube of the above medium inoculated from the fecal broth culture was incubated through 48 hours at 37°C. In the absence of growth or in the presence of growth with no motility and no change in color of the precipitate the tube was discarded. In the presence of growth, motility, and a black precipitate a loopful was streaked on the surface of a Petri plate containing lead acetate-thiosulfate agar. A thin film of the same medium was layered over the inoculated surface. Under this semianaerobic condition *B. paratyphi* produces large dark brown colonies. In the presence of such colonies transfers were made from several to lactose and saccharose fermentation tubes. In the absence of acid and gas the



cultures were confirmed by agglutination with the two type antisera. With each lot of fecal cultures a medium control inoculated from a young broth culture of *B. paratyphi* was included. The above procedure was followed in all the subsequent work.

*The Incidence of Carriers among the Breeders as Determined by Cultural and Serological Examination.*

Of the 105 guinea pigs examined 69 were added to the breeding stock after the appearance of the second infection. Only 4 were survivors of the initial outbreaks in 1924. An agglutination test with the two types of *B. paratyphi* as antigens was made on serum from each animal. Eight samples failed to agglutinate either type in the lowest dilution

TABLE II.

*Number of Samples Showing Agglutination of One or Both Types of B. paratyphi at Each Dilution Interval.*

Serum dilution.....	1:20			1:40			1:80			1:160			1:320			1:640			1:1280		
Type of bacillus.....	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both
Number of samples .....	0	11	7	3	5	5	1	22	1	1	15	1	4	12	2	2	5	0	0	0	0

employed. The remaining samples agglutinated one or both of the antigens in dilutions ranging from 1:20 through 1:640. With 66 serums of the latter group the dilution was 1:80 or higher. A detailed classification of the samples as to limiting dilution for the major agglutinin is given in Table II. Only 5 of the 24 males included in the group examined showed an agglutinin titer higher than 1:80 as compared with 45 high reactors among the 81 females. Two serums which agglutinated one type through a dilution of 1:160 failed to agglutinate the other type in 1:20. The majority of serums, however, agglutinated both types generally with a difference of one or two dilutions between them. With 70 samples the major agglutinin (highest limiting dilution) was for Type II antigen as compared with 11 for Type I.

*B. paratyphi* was isolated from the feces of 3 guinea pigs of the group examined.

One was an old sow added to the breeding stock in the fall of 1924 shortly after the termination of the active stage of the initial infection. Type II *B. paratyphi* was cultivated from the feces on 2 successive days. The blood serum agglutinated Type I antigen through 1:160, Type II antigen through 1:320. The animal was kept under observation for 3 weeks and then killed. The uterus contained 3 half-term fetuses. The surface of the spleen was scarred and pitted. Other organs were normal. Cultures from the spleen, placental tissue, and cecal feces were all negative.

The other positive cases were younger sows added to the breeding stock in December, 1926, and July, 1927, respectively, before and after the appearance of the second infection. The former was an active case. *B. paratyphi*, Type II, was isolated from the first fecal culture. The next morning the animal was weak with labored breathing and was killed. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:640. At autopsy a peritonitis was encountered. A heavy exudative membrane was reflected over the surface of the spleen and portions of the liver. The abdominal cavity contained a quantity of turbid fluid. Type II *B. paratyphi* was isolated from the spleen, liver, and cecal feces.

The second of the younger animals was a carrier. A Type II organism was cultivated from the feces on successive days. The blood serum agglutinated Type I antigen through 1:40, Type II through 1:80. After 2 weeks the animal was killed and autopsied. The uterus contained 2 nearly full-term fetuses. The spleen was atrophic and extensively roughened and pitted. Cultures from the spleen, placental tissue, cecal feces, and fetal spleens were all negative.

#### *The Incidence of B. paratyphi among a Group of Selected Breeders.*

All guinea pigs from the tested group which showed consecutively negative fecal cultures and with an agglutinin titer of 1:80 or lower were segregated as a nucleus for a new breeding stock. The carriers were killed, as noted. The guinea pigs which showed high agglutinin content were returned, in most instances, to their original unit and isolated in special cages. A few were killed and autopsied. The selected animals were isolated in a separate unit in groups numbering up to 5 sows and 1 boar. The two units were maintained independently.

During the 5 months occupied in testing the entire group additional fecal cultures were taken at intervals from the segregated individuals. At first, mass cultures were made from the breeding cages. During one examination which comprised eight cages a culture of Type I *B. paratyphi* was obtained from the feces of a single cage. The guinea pigs were immediately transferred to individual

pens and cultures made on 2 successive days. The positive culture was traced to a single sow from which the same type of organism was isolated. The animal was removed and later killed. At autopsy no gross lesions were encountered and cultures from the spleen, uterus, and cecum were all negative. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:20. Fecal cultures from the other inmates of the cage were all negative.

For the third fecal examination the guinea pigs were isolated in individual cages and a single culture made. The group comprised 46 adult animals distributed in ten cages. In addition there were 22 younger breeders representing the second generation of the selected population. The entire lot of fecal cultures was negative.

The segregated group was maintained in the same manner as the main breeding stock. Pregnant sows were removed to individual cages shortly before parturition. Young guinea pigs were kept with their dam for 2 to 3 weeks and then weaned. The sow was returned to her original breeding cage. The young were separated as to sex and placed in large open pens. A few of the weaned young were used for experimental work. 9 of these were carefully examined subsequent to infection with other bacteria. Cultures from the entire spleen were negative throughout. The majority of them were allowed to mature for breeding purposes. Before admission to the breeding stock they were isolated in individual cages and fecal cultures made.

The selected population was kept under observation and all animals that died were autopsied and cultured. Through June, 1928, a total of 111 guinea pigs was examined. The majority of them were still-born or unweaned young. *B. paratyphi* was isolated from the spleen on four occasions.

The first culture was obtained in February from a female stock guinea pig. At autopsy a large amount of turbid fluid was found in the pleural and peritoneal cavities. The spleen was enlarged and coated with a heavy, tenacious exudate which was likewise reflected over portions of the liver. The cervical and peritoneal lymph nodes were enlarged and congested. Type I *B. paratyphi* was isolated from the spleen, gall bladder, pleural fluid, and cecum.

The second culture was isolated from an unweaned male guinea pig whose dam had died on the previous day. At autopsy the lymphoid tissue of the cecum was congested and a culture of Type II *B. paratyphi* was secured from the spleen. The dam showed no evidence of infection. The spleen failed to show *B. paratyphi*.

The third and the fourth cultures were isolated in June, in both cases from sows. At autopsy, the first animal showed an enlarged and congested spleen. Both spleen and liver were coated with a tenacious exudate. The gall bladder contained purulent fluid. There was an inflammation of the small intestine. The abdominal cavity contained a mucoid fluid. Type II *B. paratyphi* was isolated from the spleen. The second sow was carrying 4 nearly full-term fetuses. No gross manifestations of infection were encountered. Type I *B. paratyphi* was cultivated from the spleen.

*The Incidence of B. paratyphi among Unweaned Young of the Main Population.*

A less extensive study was made of the incidence of carriers among the unweaned young of the main population.

Monthly examination of 6 guinea pigs from as many litters, chosen at random, was carried out during the period between September, 1927, and February, 1928. The animals varied in age from 2 to 3 weeks. They were brought to the laboratory, bled from the heart under ether, and killed. Postmortem examination was made and cultures taken from the spleen, gall bladder, small intestine at a Peyer's patch, and cecal feces.

During October, 2 fecal carriers and 1 active case of paratyphoid were encountered. All 3 were females, 3 weeks old. Type II *B. paratyphi* was isolated from the cecum of the 2 carriers. The abdominal organs showed no gross changes. Cultures from the spleen, gall bladder, and small intestine were negative in each instance. The active case showed typical gross changes in the abdominal viscera. The spleen was enlarged, congested, and showed a single nodular focus. The liver showed a few small foci. There were numerous exudative plaques on the surface of the cecum with foci in the lymphoid tissue at the head. Type II *B. paratyphi* was obtained from the spleen, small intestine, and cecal feces. During the same month 3 active cases occurred among the young of a second sow from the same breeding cage. In February and March fatal cases were noted among the young of 2 sows originally caged with the dam of the first carrier, mentioned above. All of the unweaned guinea pigs examined during the remaining months were normal with negative cultures throughout.

Agglutination tests were made on blood samples from all the unweaned guinea pigs. The procedure previously outlined was followed except that the dilution ranged from 1:10 through 1:640. The results expressed in terms of the limiting dilution showing agglutination are given in Table III. Seven samples failed to agglutinate either type of antigen in the lowest dilution. Four samples agglutinated both types through the same limiting dilutions. The remainder agglutinated both types of antigen but the limits of reaction varied by one or more dilutions. With eighteen samples of this number the major reaction was against Type II, while with seven the major reaction was against Type I. Classified according to sex it may be noted that serums from 2 of the 12 males and 5 of the 24 females were negative. Serum from the first of the fecal carriers agglutinated Type I through 1:20, Type II through 1:80; that from the second agglutinated Type I through 1:20, Type II through 1:40. There was no agglutination against either type with serum from the active case.

Agglutination tests were also made on serum from the dams of 3 unweaned guinea pigs and from the remaining young of their litters. The findings, which suggest maternal transmission of antibodies to the suckling young, are given in

TABLE III.

*Limiting Agglutination with Serum from Unweaned Guinea Pigs.*

Month	Type of bacillus	Limiting dilution of serums					
Guinea pig No.....		1	2	3	4	5	6
September	I	40	20	40	80	20	10
	II	80	40	40	40	80	20
October	I	20	20	20	20	—	—
	II	20	80	40	80	—	—
November	I	20	40	20	40	20	20
	II	80	20	80	10	80	160
December	I	80	40	80	10	—	—
	II	20	80	40	20	—	10
January	I	—	—	10	—	10	10
	II	—	—	20	—	20	20
February	I	20	20	—	10	80	20
	II	80	—	—	10	20	20

TABLE IV.

*Agglutinin Titer of Serum from Sows and Their Unweaned Young.*

Class of guinea pig	Limiting dilution	
	Type I	Type II
1st young.....	10	10
Sow.....	80	20
2nd young.....	20	10
3rd ".....	20	10
1st ".....	80	20
Sow.....	160	20
2nd young.....	40	10
3rd ".....	40	10
1st ".....	20	80
Sow.....	40	320
2nd young.....	20	80
3rd ".....	20	80

Table IV. It may be said that all the animals were later killed with negative pathological and bacteriological findings throughout. There was an interval of 4 days between the examination of the first young and the other guinea pigs of the group.

#### DISCUSSION.

The examination of breeders described in the foregoing pages was begun in October, 1927. 13 months earlier the second type of *Salmonella* infection had appeared spontaneously in the population. There had been a slow but general spread of the second bacterium throughout the breeding group. Specific deaths occurred among the sows or the unweaned young of sows from 32 of the 48 cages. The Type I infection had long since become endemic, with the mortality rate in general on a low level.

The serological findings appear to reflect the distribution of the two *Salmonella* types. 92 per cent of the 105 serums tested showed well defined agglutination against one or both types of *B. paratyphi* in dilutions ranging from 1:20 through 1:640. With 86 per cent of the reactors the higher agglutination was against the second, more recent type of organism. With 13 per cent the higher agglutination was against the initial form. In each case the majority of serums agglutinated both types although one in lower dilution. The physical form of the sediment was not sufficiently defined to permit a distinction between common agglutinins for the one type and specific agglutinins for the other.

It was assumed that the high agglutinin content of individual serums was referable to a multiplication of one or both strains in the system of the guinea pig. Postmortem cultures from a small series of high reacting guinea pigs failed, however, to demonstrate the specific organism in the spleen, liver, or section of the small intestine. These observations suggest that a high agglutinin titer may be maintained in the absence of a persistent localization of *B. paratyphi* in the more usual sites. The bacteriological examination, however, was not exhaustive. The entire spleen was cultured but examination of the liver and small intestine was necessarily limited. Moreover, the possible localization of the specific bacterium in regional lymph nodes is not ruled out. Temporary colonization of *B. paratyphi* in one or another of these sites might well impart the stimulus leading to antibody formation.

The serological findings with the unweaned guinea pigs roughly parallel those with the breeders. The total number of reactors while high was less than with the mature animals, 80 per cent as compared with 92 per cent. The young guinea pigs also showed a greater number of high reactors against the second type of *B. paratyphi* than against the first. The difference was not as marked, however, as with the older animals. The agglutinin titer in general was on a lower level than that of the breeders. The highest limiting dilution recorded was 1:160, with the majority of serums well below that figure. A number of serums from the breeders agglutinated through 1:640 with a fair proportion showing reaction between 1:80 and 1:320. There was a suggestion that the agglutinin content of the young was referable, in part, to placental transmission or to lactation. Sublethal infection at birth with immunization was not excluded as a possible cause of the presence of agglutinins in the group at large.

By way of comparison it may be of interest to note the serological findings reported by Amoss (4) on the survivors from an artificially induced paratyphoid epidemic in mice. A population of 300 mice comprising susceptible individuals in contact with a previously infected group was set up. During the epidemic wave which followed, 69 per cent of the population died. He tested the serum from 56 of the survivors for agglutinin against the causal organism, Mouse Typhoid II. The serum from 37 or 66 per cent partially agglutinated the organism in dilutions ranging from 1:20 to 1:160. The serum from 27 showed no agglutination in 1:20.

The cultural tests in the present work indicated a low incidence of fecal carriage among the breeders. *B. paratyphi* was isolated from the feces of only 3 sows, 1 of them an active case. A group of 50 guinea pigs selected from the total number examined was again cultured on three occasions with the detection of a single additional carrier. While the results cannot be regarded as strictly quantitative it is believed that the figure cited is a fair index of the extent of specific fecal excretion in the breeding group as a whole. Some of the animals examined were selected from cages in which fatal cases of paratyphoid had previously occurred. Conditions were favorable for the ingestion of the specific bacteria with food soiled by fecal excretions. In the face of such conditions the small number of fecal carriers actually detected

seems to bear out the suggestion made in a previous paper that specific fecal excretion with adult guinea pigs is of relatively short duration (5).

Examination of the unweaned young showed that fecal carriage of *B. paratyphi* may occur with nursing guinea pigs in the absence of generalized infection. *B. paratyphi* was isolated from the feces of 2 guinea pigs which showed no manifestations of disease. The group tested was too small to indicate the actual extent of such carriage. The young guinea pigs after weaning are transferred to the general stock for laboratory use. The presence of fecal carriers among them may account for the subsequent occurrence of sporadic cases or carriers. It is known that *B. paratyphi* may localize in the spleen or liver with no indication of multiplication or injury. It is possible that the introduction of other bacteria into such guinea pigs may be followed by multiplication of the paratyphoid types with active disease.

Both types of *B. paratyphi* appeared among the guinea pigs of the segregated group. The selected population which included breeders and later unweaned young and stock was maintained in a separate unit. There was no communication with the main supply of guinea pigs. Chance contact with field animals was practically impossible. Introduction of the specific bacteria with the food was not entirely ruled out. It seemed more probable, however, that the initial selection was not sufficiently rigid and that a number of spleen or fecal carriers were admitted to the new breeding stock. To date (June, 1928) there has been no evidence of extensive dissemination of the bacteria throughout the population.

#### SUMMARY.

A group of 105 breeders and 36 unweaned guinea pigs was tested to determine the extent of specific fecal excretion and the proportion of serum reactors in a population naturally infected with two types of *B. paratyphi*. The second, more recent type of organism was isolated from the feces of 3 breeders and 3 young. No carriers of the first type were detected. 86 per cent of the breeders and 72 per cent of the unweaned guinea pigs agglutinated the second type of *B. paratyphi*



in dilutions ranging from 1:10 through 1:640. 13 per cent of the breeders and 28 per cent of the unweaned young agglutinated the initial type. There was a cross or double agglutination in most instances. The serological findings roughly reflected the distribution of the two types as indicated by the mortality rate of the population at large.

50 breeders selected on the basis of agglutination and fecal examination and therefore supposedly free from infection were segregated and kept under close observation. Both types of *B. paratyphi* subsequently appeared in the group.

During this time carriers were discovered by others in the Department among the stock guinea pigs used for other experiments in that cultures of the entire spleen were positive in perhaps 5 to 10 per cent of the guinea pigs so used.

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## OBSERVATIONS ON FLAGELLAR AND SOMATIC AGGLUTINATION.

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In a recent review Hadley (1) presented a critical discussion of the flagellar antigen concept as originally proposed by Smith and Reagh (2) to account for the difference in agglutination between motile and non-motile strains of the hog cholera bacillus. He questioned the validity of the hypothesis on the ground that the particular order of serologic reaction was not limited to bacterial species characterized by the possession of flagella. He suggested that such serologic differences might eventually come to be studied in terms of the presence or absence of certain specific soluble substances wherever their point of origin in the bacterial cell. Some work done by the writer on the agglutinability of deflagellated motile bacteria seemed to have a bearing on the latter suggestion. The work was extended somewhat and is discussed in the present paper.

The initial work of Smith and Reagh (2) was concerned with the agglutinative affinities of two strains of the hog cholera bacillus, one motile, the other a non-motile variant. A "motile" antiserum agglutinated the motile strain in high dilution with a floccular type of clump. Microscopically the clumped bacilli were separated by narrow spaces. It agglutinated the non-motile strain in a lower dilution with granular clumps in which the bacteria were not separated by any appreciable space. A "non-motile" antiserum agglutinated both strains in relatively low dilution with granular clumping. The non-motile strain removed the granulating but not the flocculating agglutinin from "motile" antiserum. The motile strain reduced the granulating agglutinin of the "non-motile" serum. The writers concluded that the agglutinins for the flagella and for the body of the bacilli, at least so far as the large group of pathogenic colon derivatives were concerned, were distinct, not mutually interacting substances.

These observations were subsequently extended by other workers among whom the following may be mentioned. Beyer and Reagh (3), working under Smith,

showed that flagellar antigen and somatic agglutinin were heat-labile at 70°C. while somatic antigen and flagellar agglutinin were heat-stable. Orcutt (4) working with the hog cholera bacillus and Balteanu (5) with the vibrio comma demonstrated the agglutinative and antigenic properties of pure flagellar suspensions. Yokota (6) carried out similar studies on flagellar suspensions from *B. typhosus* and also on the bacteria deflagellated by shaking. Goyle (7) compared the agglutinative relationships of heated and untreated suspensions of *B. typhosus* and *B. enteritidis* and their variants. Arkwright (8) described the microscopic findings with floccular and granular agglutination.

The multiple antigen hypothesis as related to bacteria has also been criticized by Tulloch (9) in an extended series of papers. He pointed out certain irregularities which cannot be cited here.

*Direct Agglutination of Whole, Shaken and Heated Antigens by "Whole" Antiserum.*

Two species of Salmonella designated *B. paratyphi* Types I and II were employed in the present work.

The strains used were originally cultivated from spleen tissue. They were isolated during the course of a natural epidemic in a guinea pig population (10). The final cultures represented the growth from single colonies replated three times on agar. They were normal smooth strains as to their growth in broth and their colony formation on agar, were actively motile and gave a characteristic agglutination with diagnostic antisera then in use.

Fresh antisera against the whole bacteria were prepared in rabbits.

The growth from 18 hour agar cultures was removed with physiological salt solution, centrifuged, the packed bacteria washed once, resuspended in 0.2 per cent formalinized saline and heated to 56°C. for 1 hour. Five intraperitoneal injections at 3 day intervals were given. The rabbits were previously tested for normal or immune agglutinins active for the particular antigens. The two type antisera, termed "whole," were tested against whole, heated and shaken suspensions of both types of *B. paratyphi*. The suspensions were prepared as follows: The growth from a Blake bottle was removed with 10 cc. of saline, washed once, resuspended in saline and divided into three portions. One was untreated. One was heated to 100°C. for 30 minutes in a water bath, washed once after centrifuging and resuspended in saline. One was shaken for 1 hour in a mechanical shaker, washed twice after centrifuging and resuspended in saline. The three suspensions were finally standardized to equal opacity, 2.4 on the Gates scale. In testing, 0.5 cc. amounts of diluted serum and suspension were mixed. The final dilutions were doubled at each interval from 1:100 through 1:51,200. The

tubes were incubated at 37°C. for 3 hours and read after overnight refrigeration. The limits of agglutination with each serum against the six antigens are given in Table I. The limit of agglutination is defined as the highest dilution showing any macroscopic evidence of clumping after approximately 24 hours.

The agglutination of the several antigens with reference to the character of the sediment and the limiting dilution was much the same with both antisera. The whole antigen was agglutinated in high dilution by its homologous serum. The heated and shaken antigens were agglutinated equally through a much lower limit. With the

TABLE I.

*Direct Agglutination of Whole, Shaken and Heated Antigens with Type I and Type II Antisera.*

Serum	Antigen	Limiting dilution	Type of agglutination
Type I "whole"	Type I whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" II whole	1:800	Atypical granular
	" " shaken	1:800	Granular
	" " heated	1:800	"
Type II "whole"	" II whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" I whole	1:200	Atypical granular
	" " shaken	1:200	Granular
	" " heated	1:200	"

antisera of opposite type the three antigens were agglutinated equally in still lower dilution. The reaction with the homologous whole antigen was predominantly floccular throughout with an admixture of granular clumps in the lower dilutions. The reaction was rapid with the formation of coarse clumps which on settling formed a light feathery sediment. In the lower dilutions the supernatants were clear or nearly so. Microscopically the sediment was composed mainly of large, poorly defined clumps with an open work appearance. With the lower dilutions there were also smaller compact clumps which were more sharply defined. The type of agglutination with the ho-

mologous heated and shaken antigens was granular throughout. The reaction was retarded. The sediment varied from an irregular, wrinkled, compact disc in the lower dilutions to a granular mold fitting the rounded portion of the tube in the higher dilutions. The clumps were not easily broken up on shaking. The supernatants tended to be clear up to the last two dilutions with a graded sediment. With the antigens of the opposite type the agglutination was granular. The macroscopic picture with the heated and shaken antigens was identical with that described above. The character of the sediment with the whole antigens was somewhat different. The Type II antigen formed a compact, even, button-like sediment with a practically clear supernatant. The Type I antigen formed a sediment closely approaching the typical granular form except that the clumps were larger and not as closely packed. Microscopically the clumps were of the granular type in both cases.

*Absorption of "Whole" Antiserum with Whole, Shaken and Heated Antigens.*

The two "whole" antisera were absorbed with the three homologous antigens.

In absorbing 0.25 cc. of packed bacteria, 0.1 cc. of serum and 2.4 cc. of 0.2 per cent formalized saline were employed, an absorbing dose and a serum dilution of approximately 1:10 and 1:25 respectively. The absorbing antigens were prepared as previously described and sedimented for 1 hour in graduated centrifuge tubes to a constant volume. The final antigen-serum mixtures were incubated at 37°C. for 3 hours with frequent shakings. After overnight refrigeration they were centrifuged and the clear supernatants tested. The dilution series previously described was employed. Unabsorbed serum similarly diluted with formalized saline was subjected to the same incubation and tested with the same antigen suspension. The results were identical with those given in Table I. The limiting agglutination of the three antigens by the absorbed sera is given in Table II.

After absorption with whole antigen both sera continued to give a floccular reaction with the homologous antigen in very low dilution. There was no reaction with the shaken and heated antigens in the lowest dilution employed. Absorbed with shaken and heated antigens both sera agglutinated the homologous whole antigen through dilution 1:12,800, one dilution removed from the limit of agglutina-

tion with the unabsorbed serum. The type of clumping was purely floccular in all cases. The supernatants showed a diffuse turbidity which was graded. There was no agglutination of the shaken and heated antigens in the lowest dilution. The removal of a considerable amount of flocculating agglutinin by the treated antigens was indi-

TABLE II.

*Agglutination Limits with Type I and Type II Antiserums after Absorption with Whole, Shaken and Heated Antigens.*

Serum	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I whole	Type I whole	1:400	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
Type II whole	Type II whole	1:200	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated

cated. Further evidence bearing on the absorption of flocculating agglutinin by the treated antigens will be presented in another paper.

*Agglutination with Shaken and Heated Antiserums before and after Absorption.*

The immunizing properties of the shaken and heated suspensions of the motile bacteria were determined.

The suspensions were prepared as described. The treated and washed bacteria were sedimented in the centrifuge, resuspended in 1 per cent formalinized saline and kept for 3 days in the refrigerator. They were resedimented, washed twice and resuspended in 0.2 per cent formalinized saline. It was necessary to kill the shaken bacteria and the above method was chosen in preference to heat at 56–58°C. on successive days. To keep the suspensions uniform both were subjected to the same conditions although the heated suspension (100°C. for 30 minutes) was obviously inactivated. Subcultures made on consecutive days were sterile. A rabbit was immunized intraperitoneally with each suspension. Five injections at 3 day intervals were given with the Type I antigens, four injections with the Type II. 7 days after the last injection the animals were bled and killed. At autopsy no focal lesions were detected in any of the usual sites: spleen, liver or lymphoid tissue of the intestinal tract. There were traces of exudate on one or another of the visceral organs. Walled-off accumulations of purulent material along the cecum were commonly observed. Cultures from spleen, liver, purulent material and cecal feces failed to show *B. paratyphi*. The agglutinin content of the several antisera was determined by direct agglutination and by absorption employing whole, shaken and heated antigens of the same type. The limits of agglutination of the shaken and heated antisera before and after absorption are given in Table III.

The shaken and heated antisera agglutinated the three homologous antigens equally. The limiting dilutions were higher with the Type I sera as might be expected in view of the longer immunization. In both cases the clumping of the shaken and heated antigens was typically granular. The sediment with the whole antigens was granular microscopically. Macroscopically the sediment with each type was identical in appearance with that described previously for whole antigen agglutinated by serum of opposite type.

The absorption tests gave further evidence for the absence of flocculating agglutinin. Absorption with any one antigen removed most of the agglutinin for all three suspensions. Irregularities in the titer were noted but had no bearing on the presence or absence of flocculating agglutinin. In general the reduction of agglutinin was a little less complete than that previously noted with the whole antisera. Although the shaken and heated antigens were able to remove some flocculating agglutinin from whole antiserum, there was no indication that they were able to produce the same upon injection in rabbits.

TABLE III.

*Agglutination Limits with Shaken and Heated Serums of Both Types before and after Absorption.*

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I shaken	1:6,400	Type I whole	Type I whole	1:200 1:200 1:200	Type I whole " " shaken " " heated
		Type I shaken	Type I shaken	1:200 1:200 1:200	" " whole " " shaken " " heated
		Type I heated	Type I heated	1:200 1:200 1:200	" " whole " " shaken " " heated
	1:6,400	Type I whole	Type I whole	1:200 1:200 1:100	Type I whole " " shaken " " heated
		Type I shaken	Type I shaken	1:200 1:200 1:200	" " whole " " shaken " " heated
		Type I heated	Type I heated	1:200 1:200 1:100	" " whole " " shaken " " heated
	1:3,200	Type II whole	Type II whole	<1:100 <1:100 <1:100	Type II whole " " shaken " " heated
		Type II shaken	Type II shaken	1:100 1:100 1:100	" " whole " " shaken " " heated
		Type II heated	Type II heated	1:100 1:100 1:100	" " whole " " shaken " " heated
Type II heated	1:3,200	Type II whole	Type II whole	1:200 1:200 1:200	Type II whole " " shaken " " heated
		Type II shaken	Type II shaken	1:200 1:200 1:200	" " whole " " shaken " " heated
		Type II heated	Type II heated	1:100 1:100 1:100	" " whole " " shaken " " heated
	1:3,200	Type II whole	Type II whole	1:200 1:200 1:200	Type II whole " " shaken " " heated
		Type II shaken	Type II shaken	1:200 1:200 1:200	" " whole " " shaken " " heated
		Type II heated	Type II heated	1:100 1:100 1:100	" " whole " " shaken " " heated



*Agglutination of Untreated, Shaken and Heated Suspensions of a Non-Motile Bacterium.*

The agglutination tests were repeated with a culture of *Staphylococcus aureus* and its homologous antiserum to afford a basis for comparing the agglutinative affinities of a non-motile organism. The staphylococcus was recently isolated from a skin abscess and was a normal, smooth strain. A rabbit antiserum against the whole antigen was prepared. The same procedures outlined for the motile bacteria were followed in immunization, preparation of antigens and performance of the agglutination tests. The limits of agglutination before and after absorption with whole and heated antigens are given in Table IV.

TABLE IV.

*Limits of Agglutination with Staphylococcus Antiserum before and after Absorption*

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Staphylococcus whole	1:1,600 G*	Whole	Whole	1:50 G	Whole
			"	1:50 G	Heated
	1:1,600 G	Heated	Heated	1:50 G	Whole
			"	1:50 G	Heated

\* Granular clumping.

The shaken antigen showed the same limiting dilution upon direct agglutination and was not employed in absorption.

The staphylococcus antiserum agglutinated whole and heated antigens to the same titer limit. The reactions were retarded, the limiting dilutions were relatively low and the clumping was typically granular both macroscopically and microscopically. The titer of the serum, 1:1,600 after five injections, was not noticeably increased by three additional injections. The agglutination of whole and heated antigens by the "non-motile" antiserum was identical in character with that of heated and shaken antigens by the "motile" antisera.\* The whole and heated staphylococcus antigens possessed approximately the same absorptive capacity for agglutinin as indicated by partial absorption. Reciprocal absorption with a "heated" staphylococcus antiserum was not made.

*The Origin of Soluble Material Extracted by Heat from the Motile Bacteria.*

Some additional experiments bearing on the production of soluble material from the motile bacteria may be cited. A considerable decrease was noted in the volume of packed bacteria after heating to 100°C. for 30 minutes. Goyle (7) had previously called attention to the decreased opacity of heated bacterial emulsions. The supernatants from such heated suspensions after sedimentation of the bacteria were distinctly milky in appearance and when mixed with "whole" antiserum gave a precipitate in moderate dilution. Specific soluble material precipitable with immune serum had evidently been extracted from the bacteria at the temperature employed. Happold (11) has recently described a precipitinogen present in the filtrates of steamed broth cultures of *B. aertrycke* (mutton).

Comparative tests were made on the supernatants from heated whole suspensions and heated shaken suspensions to determine the effect of the presence of flagella on the amount of extracted soluble material. Filtrates of the growth from 18 hour agar cultures of the two motile bacteria were included.

Blake bottles were inoculated with 2.5-3 cc. of 6 hour broth cultures of the bacteria and incubated at 37°C. for 18-24 hours. The growth from each bottle was removed with 5 cc. or so of saline, transferred to a graduated tube and centrifuged for 1½ hour. The supernatant was withdrawn and the packed bacteria washed once with 8 cc. of saline. Both supernatants were saved and later filtered through medium Berkefeld candles. For the final suspensions 1 cc. of saline was added for each 0.1 cc. of packed bacteria and the cells resuspended. One suspension of each type was shaken for 1 hour. The packed bacteria, after centrifuging, were washed three times and finally resuspended in saline according to the same ratio. The untreated and the shaken suspensions of each type were then heated to 100°C. for 1 hour in a water bath. After centrifuging the supernatants were removed and filtered through Berkefeld filters.

The final solutions prepared according to the above methods all had a milky, opalescent appearance. There was no deposit upon standing. The culture supernatants were perfectly clear but showed a yellowish tinge. The volume of packed bacteria before heating was approximately 1 cc. with Type I, 0.7 cc. with Type II. After heating, the volumes were 0.7 cc. and 0.4 cc. respectively. The suspensions were centrifuged for 1 hour at the same speed in both cases. The culture and suspension filtrates were tested with "whole" antiserum of the same type. Only one culture filtrate was employed with the Type II *B. paratyphi*.

In testing, the antigens were diluted in series and the serum kept constant, 0.5 cc. amounts of the former and 0.1 cc. amounts of the latter being employed. The tubes were incubated at 37°C. for 1 hour, followed by overnight refrigeration. The final readings are given in Table V. The first culture filtrates and the suspension filtrates showed a ring reaction with beginning precipitation in the lowest dilutions after 1 hour. Upon standing there were decreasing amounts of a granular sediment, indicated in the table by plus signs. The highest dilutions showed a distinct clouding of the supernatant but no sediment. No bacteria were detected upon microscopic examination.

TABLE V.

*Precipitin Tests with Culture Filtrates and Heated Suspension Filtrates of the Two Salmonella Types.*

Antigen	Serum	Serum dilutions								
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Type I	Type I whole									
1st culture filtrate		+	+	±	Cl.*	S.cl.*	-	-	-	-
2nd " "		S.cl.	-	-	-	-	-	-	-	-
Heated whole suspension		++	++	+	+	±	±	Cl.	S.cl.	-
" shaken "	Type II whole	++	++	+	+	±	±	Cl.	S.cl.	-
Type II										
1st culture filtrate		+	±	±	S.cl.	-	-	-	-	-
Heated whole suspension		++	++	++	+	±	±	Cl.	S.cl.	-
" shaken "		++	++	++	+	±	±	Cl.	S.cl.	-

\* Cl. = cloudy supernatant; S.cl. = slightly cloudy supernatant.

The above tests indicate the presence of small amounts of soluble precipitate material in the diluted culture filtrates of both *Salmonella* species. The amount was greatly reduced by one washing as indicated by the reaction with the second Type I culture filtrate. Similar material was extracted from the bacteria by heat and was present in considerably greater amount in the diluting medium. The supernatant from the heated whole bacteria reacted quantitatively the same as that from the heated shaken bacteria. The amount of soluble material present in the fluid as a result of flagellar disintegration was evidently too small to affect the titer. It may be said, too, that the

agglutinability of the bacteria was not greatly influenced by the loss of the somatic constituents extracted by heat.

#### DISCUSSION.

The experimental work on agglutination reported in the previous section is in no sense original. The general conformation of the observations to the early flagellar hypothesis of Smith and Reagh (2) and its extension by others is the main reason for its presentation. The observations bear out the altered agglutinability of motile bacteria following shaking and heating at 100°C. Both treatments result in deflagellation of the bacteria. With the former the flagella are mechanically broken off and subsequently removed by washing. With the latter a disintegration of the flagella occurs and the soluble products are removed upon washing. Tulloch (9) has objected to the application of heat for demonstrating separate antigens on the ground that the physical nature of the bacterial cell as a whole may undergo a change resulting in altered agglutinability. The results following deflagellation by shaking are less easily disposed of in this way. No marked change in the physical state of the cell is apparent, aside from the removal of one morphological structure, the flagellum. The shaken bacteria are viable and capable of normal growth upon transfer to media. In common with the heated bacteria, however, they are incapable, in the present case, of producing flocculating agglutinin upon injection in rabbits. Admittedly there is lack of agreement concerning the true antigenic nature of deflagellated bacteria. In some instances at least the reported ability of treated bacteria to produce flocculating agglutinin appears referable to the presence of flagellar material in the suspension used for immunization.

One exception to the flagellar hypothesis was noted with the present work. The shaken and heated suspensions were able to absorb a certain amount of flocculating agglutinin from "whole" antiserum. It is suggested that the anomalous reaction may be attributed to a non-specific adsorption of antibody. Regarding the reaction in an animal host as the more exacting criterion it appears that the motile bacteria have lost in immunizing ability with the loss of flagella. This in turn implies that the substance of the flagella embraces a specific antigen.

The objections of Hadley (1) to such an assumption have been previously noted. He intimates that serological differences similar to those noted with whole and deflagellated bacteria may be explained by the presence of soluble specific substances disregarding particular morphological elements.

A small amount of soluble material precipitable with immune serum was found present in culture medium supporting the growth of the motile bacteria. The suspensions used for the production of "whole" antiserum were carefully washed and the amount of such material reduced to a practically negligible quantity. The resulting antiserum, however, contained both granulating and flocculating agglutinin. If a soluble substance were responsible for the appearance of flocculating agglutinin the bulk of it must have been produced from the bacteria after their introduction into the animal host. The "shaken" and "heated" antisera contained only granulating agglutinin. Hence if a soluble substance is to be regarded as the antigen leading to the production of flocculating agglutinin it seems necessary to associate it with the flagella. Orcutt (4) has shown that pure flagellar suspensions (*hog cholera bacillus*) give a floccular agglutination with "whole" or flagellar antiserum. If the suspension is heated to 70°C. for 30 minutes the flagella are broken up. The floccular agglutinability is lost but on animal injection the heated suspension produces flocculating agglutinin. Apparently the flagella go into solution. With our experiments such heated suspensions in moderate dilution (1:80) gave a granular precipitate with "whole" antiserum. One might regard this reaction as the analytical production of soluble specific material which was antigenic in the sense that it could still produce antibody but which because of a change in the physical state of its precursor, the flagellum, showed altered agglutinability. Injected bacteria must eventually undergo disintegration probably with the production of similar soluble materials. Viewed in this light the suggestion of Hadley seems plausible with this modification, however, that the soluble substance be limited as an antigen to the flagella.

Hadley (1) has also questioned the purity of the flagellar suspensions used by Orcutt (4) and Balteanu (5). He suggests that the heat-labile factor present in such suspensions may equally well represent

soluble substances from the bacteria themselves. We have shown that the bulk of soluble precipitable material extracted *in vitro* from motile bacteria or produced *in vivo* in smaller amounts during growth originates in that portion of the bacterial cell which as an antigen gives rise to granular agglutinin. In combination with "whole" antiserum this material gives a precipitate which is likewise granular in nature. Its presence in flagellar suspensions could not be held accountable for the floccular reaction which occurs with "whole" or flagellar antisera.

#### SUMMARY.

Whole, shaken and heated suspensions of two *Salmonella* species were compared as to agglutinability, absorptive capacity and antigenic properties. The results were in general agreement with the flagellar antigen concept of Smith and Reagh. The removal of flagella by shaking or heating (100°C.) resulted in altered agglutinability manifested by failure to give a floccular reaction with "whole" antiserum. The deflagellated bacteria were able to absorb some flocculating agglutinin from that serum. They were unable, however, to produce flocculating agglutinin upon injection in rabbits.

Untreated, shaken and heated suspensions of a non-motile bacterium (*Staphylococcus*) showed no differences with respect to agglutinability or absorptive capacity.

Soluble precipitable material was found present in small amount in culture filtrates of the motile bacteria and in greater concentration in filtrates of heated suspensions. The bulk of the soluble material was of somatic origin and was not appreciably increased by the presence of flagella. It was possible, however, to demonstrate soluble material in heated flagellar suspensions. The relation of such soluble substances to floccular agglutination and the production of flocculating agglutinin as suggested by Hadley is discussed.

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## THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA.

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The flagellar antigen hypothesis of Smith and Reagh (1) assumes that motile bacteria as antigens give rise to two distinct types of agglutinin, termed flagellar and somatic agglutinin respectively. The former is produced by the flagella and reacts with them, whether attached to the cell or free, to give a floccular agglutination. The latter is produced by the bacterial cell proper and reacts with whole or deflagellated bacteria to give a granular agglutination. The somatic antibody does not combine with flagella. Some experiments on the agglutinability of whole and deflagellated suspensions of motile bacteria were reported in a preceding paper (2). The performance of absorption tests, during the course of the work, suggested that it might be possible to demonstrate differences between the two agglutinins with respect to their removal from sensitized bacteria.

A number of methods have been employed with more or less success in attempts to remove antibody from combination with sensitized bacteria. The early work has been reviewed in some detail by Huntoon (3). In another paper Huntoon and Etris (4) have reported an extensive series of experiments designed to secure purified solutions of antibody, largely by removal from sensitized antigens. They worked with non-motile bacteria chiefly the pneumococcus but also the meningococcus and *B. dysenteriae* Flexner. They showed that agglutinin could be removed by suspending the sensitized antigen in 10 per cent saccharose solutions or distilled water and heating to 55-60°C. for 1 hour. They were able to remove as high as 50 per cent of the combined antibody. They found that the presence of sodium chloride interfered with the removal of agglutinin but not with the removal of bactericidal and protective antibodies. A temperature of 65°C. reduced the dissociation of antibody and 70°C. almost completely prevented it. Other experiments reported by Huntoon and Etris on the removal of protective antibody from sensitized pneumococcus have no immediate bearing on the present work.



The close association of antibodies with serum globulins suggested the application of some method of globulin extraction for the removal of agglutinin. In some preliminary work it was found that heating sensitized motile bacteria to 60°C. in the presence of 5 per cent sodium chloride solution resulted in the removal of a considerable amount of one agglutinin but little or none of the other. More detailed observations on the application of the method are reported in the following experiments.

Two species of *B. paratyphi* (Types I and II) (5) of guinea pig origin were employed in the absorption and extraction tests. The serums were from rabbits immunized with inactivated suspensions of the whole bacteria.

In the absorption tests a serum dilution of approximately 1:25 and an absorbing dose of 1:10 were employed. The bacterial suspensions were freshly prepared, washed once and centrifuged to constant volume—0.25 cc. 0.1 cc. of "whole" antiserum and 2.4 cc. of physiological salt solution were added and the packed bacteria resuspended. The absorption mixtures were incubated for approximately 5 hours at 37°C. followed by overnight refrigeration. Unabsorbed serum in the same dilution was included as a control. The sensitized bacteria were sedimented in the centrifuge and the clear supernatants saved. The packed bacteria were washed four times by resuspending in 5 cc. of saline and centrifuging. After the fourth washing the packed bacteria were resuspended in 5 cc. of 5 per cent sodium chloride solution and heated to 60°C. for 1 hour in a water bath. The mixture was shaken at frequent intervals. After a final centrifuging the supernatants were withdrawn. In several instances second and third extractions were carried out in the same manner.

The agglutinin titer of the several supernatants was determined by macroscopic agglutination tests. 0.5 cc. amounts of diluted test fluid and of bacterial suspension were employed. Whole and heated suspensions were used as antigens. The former was a fresh, untreated culture, washed, and diluted to standard opacity with saline. It represented both flagellar and somatic antigens. The latter was a fresh suspension heated to 100°C. for 30 minutes, washed and similarly diluted. It represented pure somatic antigen. The tubes were incubated for 3 hours at 37°C. and read after overnight refrigeration.

The agglutination readings with unabsorbed serum, absorbed serum, four washing fluids and three extraction fluids are given in Table I. The work was done with Type II *B. paratyphi* and its homologous "whole" antiserum.

The unabsorbed serum agglutinated the whole antigen through a

TABLE I.  
*Agglutination of B. paratyphi Type II by "Whole" and Absorbed Serum, Washings and Extraction Fluids.*

Test fluid	Antigen	Test fluid dilutions											
		2	4	8	16	32	64	128	256	512	1,024	2,048	
Unabsorbed serum	W* H	CM CG	C C	C +++	C +++	+++ ++	+++ +	+++ ±	+++ -	++ -	+	-	
Absorbed serum	W H	++M ±G	+ -	± -	- -	- -	- -	- -	- -	- -	- -	- -	
1st washing	W H	+F -	± -	- -	- -	- -	- -	- -	- -	- -	- -	- -	
2nd washing	W H	±F -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	
3rd washing	W H	±F -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	
4th washing	W H	±F -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	
1st extraction	W H	+++F -	+++ -	+++ -	+++ -	+++ -	+++ -	+++ -	++ -	+	-	-	
2nd extraction	W H	+++F -	+++ -	+++ -	+++ -	+++ -	± -	- -	- -	- -	- -	- -	
3rd extraction	W H	+++F -	+++ -	± -	- -	- -	- -	- -	- -	- -	- -	- -	

\* M = mixed agglutination; F = floccular agglutination; G = granular agglutination; W = whole antigen; H = heated antigen.

dilution of 1:1,024, the heated antigen through 1:128. The actual titer limits of the serum were 1:25,600 and 1:3,200 respectively, since an initial dilution of 1:25 was used. With the whole antigen the clumping was predominantly floccular with an admixture of granular clumps in the lower dilutions. With the heated antigen the clumping was purely granular. After absorption with the whole antigen there was a residual agglutination in low dilution. The bulk of the two agglutinins had combined with the bacteria. The first washing fluid slightly agglutinated the whole antigen in low dilution. There was no reaction with the heated antigen. The other three washing fluids in spite of progressive dilution continued to give a slight floccular reaction with whole but not with heated antigen. The first extraction supernatant gave a reduced but perfectly definite agglutination purely floccular in type through a dilution of 1:512 with the whole antigen. For comparison with the agglutinin titer of undiluted serum the initial dilution of the absorbed serum must be considered, giving a final dilution of 1:12,800. There was no reaction with the heated antigen in any dilution. A second and third extraction gave decreasing floccular agglutination with the whole antigen but no reaction with the heated.

The persistent agglutination with the washing fluids suggested that a small amount of flocculating agglutinin might be removed at room temperature. Increasing the temperature and salt content of the suspending fluid gave a marked increase in the amount removed. The method of extraction failed to remove a detectable amount of granulating agglutinin.

The above experiment was repeated with different strains of the same bacterium and with another species of *Salmonella*, *B. paratyphi* Type I. The results were always much the same. The limiting dilutions of the absorbed serum, washing fluids and extraction fluids varied somewhat. In several instances the titer of the first extraction fluid was one dilution lower. Sometimes, too, there was a slight granular sediment in the lowest dilution of the heated antigen series. The substitution of suspensions deflagellated by shaking instead of by heat gave identical results. Adequate controls were included for the effect of 5 per cent sodium chloride solution on whole antigen in the absence of serum and on heated antigen in the presence of

serum. There was no indication of spontaneous agglutination or of inhibition of granular clumping.

The extraction experiment was repeated with a sensitized non-motile bacterium. A strain of *Staphylococcus aureus* and its homologous antiserum were employed. The results are given in Table II. The unabsorbed serum agglutinated both antigens to the same titer limit, 1:1,600, with a granular type of clump. There was a slight residual agglutination after absorption with the untreated suspension.

TABLE II.

*Agglutination of Staphylococcus by Unabsorbed and Absorbed Serum, Washing Fluid and Extraction Fluid.*

Test fluid	Antigen	Test fluid dilutions							
		2	4	8	16	32	64	128	256
Unabsorbed serum	W*	CG	++++	++++	+++	++	+	-	-
	H	CG	++++	+++	++	+	±	-	-
Absorbed serum	W	±G	-	-	-	-	-	-	-
	H	±G	-	-	-	-	-	-	-
1st washing	W	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-
1st extraction	W	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-

\* W = unheated antigen; H = heated antigen; G = granular agglutination.

The washing and extraction fluids failed to agglutinate either antigen in any dilution.

The extraction method was also applied to sensitized suspensions of the two motile bacteria previously deflagellated by shaking and heating respectively. The removal of some flocculating agglutinin from "whole" antiserum by such suspensions was noted in a preceding paper (2). The absorptions together with a control of unabsorbed serum were carried out in 0.2 per cent formalinized saline. Otherwise the method of procedure was identical with that previously outlined. The results for *B. paratyphi* Type II and its homologous "whole" antiserum are given in Table III

TABLE III.  
*Agglutination of Shaken and Heated Suspensions of B. paratyphi Type II by "Whole" and Absorbed Serum, Washing and Extraction Fluids.*

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	CM	C	C	+++	+++	+++	+++	+++	++	+	-
	SH	CG	C	+++	+++	+++	+	±	-	-	-	-
	H	CG	+++	+++	++	+	±	±	-	-	-	-
Absorbed with shaken suspension	W	+++ + F	+++	+++	+++	+++	+++	+++	++	+	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-
Absorbed with heated suspension	W	+++ + F	+++	+++	+++	+++	+++	+++	++	+	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-
3rd washing of shaken suspension	W	± F	-	-	-	-	-	-	-	-	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-
1st extraction of shaken suspension	W	+++ + F	+++	+++	++	++	+	-	-	-	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-
3rd washing of heated suspension	W	+ F	±	-	-	-	-	-	-	-	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-
1st extraction of heated suspension	W	+++ + F	+++	++	++	++	+	-	-	-	-	-
	SH	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-

\* M = mixed agglutination; G = granular agglutination; F = floccular agglutination; W = whole antigen; SH = shaken antigen; H = heated antigen.

After absorption with the deflagellated suspension the flocculating agglutinin titer of "whole" antiserum was decreased by one dilution. In other words the shaken and heated suspensions had removed some flocculating agglutinin in spite of the fact that flagellar antigen was entirely lacking. The third washing fluids gave a slight floccular reaction with the whole antigen in low dilution. Both of the extraction fluids agglutinated the whole suspension with a typical floccular clumping through a dilution of 1:64 (1:1,600). There was no reaction with the heated antigen. The extraction of shaken and heated suspensions of *B. paratyphi* Type I sensitized by exposure to homologous serum gave practically the same results. The titer of flocculating agglutinin was reduced following absorption and a flocculating agglutinin was removed upon extraction. The extraction tests apparently confirm the earlier observation that deflagellated suspensions of motile bacteria may absorb some flocculating agglutinin from homologous "whole" antiserum.

The fragility of bacterial flagella suggested a possible explanation, other than that of extraction, for the removal of flocculating agglutinin from the sensitized bacteria. The flagella are particularly sensitive to heat, as demonstrated by Beyer and Reagh (6), Orcutt (7) and others. At high temperature the flagella lose their morphological identity and disintegrate. Heated flagellar suspensions while they no longer give a floccular agglutination with antiserum do give a precipitate in low dilutions indicating the presence of soluble material. Although deflagellated motile bacteria may remove some flocculating agglutinin from "whole" antiserum, the bulk of that agglutinin upon absorption by whole bacteria is taken up by the flagella. The removal of flocculating agglutinin from the sensitized bacteria by the method employed might result from a breaking up of the flagella with subsequent release of the previously combined antibody. A number of experiments were performed to demonstrate the possible relationship of flagellum destruction to agglutinin removal.

The effect of temperatures on either side of that employed in the previous extractions and of high salt concentration on the agglutinating ability of Type II *B. paratyphi* was first determined.

Heavy suspensions, about twice normal opacity, were prepared in normal saline and in 5 per cent sodium chloride solution. These were heated to 55°,

60° and 65°C. respectively for 1 hour. The heated suspensions were then diluted to normal opacity with saline and tested with "whole" antiserum. The results of the agglutination tests are given in Table IV.

There was no indication that the flagella were affected by the high salt concentration or by a temperature of 55°C. The agglutinability of the organism both in saline and in 5 per cent sodium chloride solution was practically the same as that of a normal unheated sus-

TABLE IV.

*Effect of Heat and High Salt Concentration on the Agglutinability of Type II B. paratyphi.*

Suspension	Serum dilutions								
	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200
Unheated in saline	CM*	C	++++	++++	+++	+++	++	+	-
Heated 55°C. in saline	CM	C	++++	+++	+++	++	++	+	-
Heated 55°C. in 5 per cent NaCl	CM	C	++++	+++	+++	++	++	+	-
Heated 60°C. in saline	++++M	+++	++	++	+	+	+	±	-
Heated 60°C. in 5 per cent NaCl	++++M	+++	++	++	+	+	+	±	-
Heated 65°C. in saline	++++G	+++	++	+	±	-	-	-	-
Heated 65°C. in 5 per cent NaCl	++++G	+++	++	+	±	-	-	-	-

\* M = mixed type of clumping; G = granular type of clumping.

pension. At 60° and 65°C. the destruction of flagella was plainly indicated. At 60°C. both saline and 5 per cent salt suspensions agglutinated to the titer limit of the serum but the intensity of the reaction was much reduced in all dilutions. At 65°C. the type of clumping changed from a predominantly floccular one to a purely granular one. The limiting dilution was much reduced corresponding to that of deflagellated bacteria by "whole" antiserum, as pre-

vously determined. At 65°C. complete destruction of the flagella was indicated.

If the removal of antibody were due to flagellum destruction through the agency of heat then increasing the temperature to 65°C. should result in a greater yield of flocculating agglutinin. On the other hand, reducing the temperature to 55°C. should practically eliminate the removal. A number of extractions were carried out at

TABLE V.

*Effect of Varying Temperature and Salt Concentration on the Removal of Agglutinin from Sensitized Type II B. paratyphi.*

Method of extraction	Type of antigen	Limit of agglutination	Form of clumping
55°C. in 5 per cent NaCl	W*	1:128	Floccular
	H	<1:2	
55°C. in saline	W	1:64	Floccular
	H	1:2	Granular
60°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
60°C. in saline	W	1:256	Floccular
	H	1:2	
65°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
65°C. in saline	W	1:256	Floccular
	H	<1:2	

\* W = whole antigen; H = heated antigen.

various temperatures and salt concentrations of the diluent. The agglutinin titer of the several extracts is given in Table V. The results are expressed in terms of the highest dilution showing any macroscopic evidence of agglutination.

Heating the sensitized bacteria to 55°C. resulted in the removal of considerable flocculating agglutinin. As noted in Table IV there was no destruction of flagella at that temperature. The yield of free agglutinin was somewhat greater in the presence of 5 per cent sodium



chloride than in saline. With the latter diluent a small amount of granular agglutinin was removed. At 60°C. the yield of floccular agglutinin was increased with both diluents but there was no further increase at 65°C. The complete destruction of flagella at that temperature was previously noted. From the reported observations the removal of flocculating agglutinin was obviously not dependent solely on the destruction of flagella either through the agency of heat or high salt concentration.

The substitution of distilled water for salt solution in the method of extraction likewise resulted in some liberation of agglutinin. The reaction presented a number of peculiarities concerning the mode of action of the extracted antibody. Experiments bearing on the reaction are being continued.

#### DISCUSSION.

The preceding experiments indicate that the two agglutinins present in "motile" antiserum display certain differences with respect to their removal from sensitized antigen. It was found that the flocculating or flagellar agglutinin could be freed from its combination with antigen over a considerable range of temperature and salt concentration. Suspension of the sensitized bacteria in a 5 per cent solution of sodium chloride followed by heating to 60°C. for 1 hour was particularly favorable for the removal of flocculating agglutinin. The method of titration was not strictly quantitative but the removal of approximately 50 per cent of the combined agglutinin was indicated. With similar treatment there was little or no removal of granulating agglutinin either from sensitized motile or non-motile bacteria.

It may be said that the combining or clumping properties of the granulating agglutinin of "motile" antiserum are not affected by the conditions imposed upon it during extraction. "Motile" antiserum diluted with 5 per cent sodium chloride solution and heated to 60°C. for 1 hour was found to agglutinate heated homologous antigen in as high dilution as did the untreated serum. The content of granulating agglutinin was not noticeably reduced.

Huntoon and Etris (4) had previously claimed that the presence of salt interfered with the removal of agglutinin from sensitized antigens. Their work was confined to the pneumococcus, the menin-

gococcus and *B. dysenteriae* Flexner. All of these organisms are non-motile and as antigens give rise to granulating agglutinin but not to flocculating agglutinin. With the foregoing experiments the presence of sodium chloride was found to inhibit the removal of granulating agglutinin from sensitized motile and non-motile bacteria. Increasing the concentration of sodium chloride, on the other hand, increased the amount of flocculating agglutinin removed from the sensitized motile bacteria. A 5 per cent solution of sodium chloride gave a greater yield than did an 0.85 per cent solution. The present findings confirm the work of Huntoon and Etris on the inhibitory effect of salt on the removal of that agglutinin which reacted, in their case with non-motile bacteria, and in the present case with non-motile and deflagellated motile bacteria. Their original statement must be modified, however, to include only granulating agglutinin, since the presence of salt favors the removal of flocculating agglutinin.

It was shown that destruction of flagella through the agency of heat with a subsequent liberation of combined agglutinin could not alone explain the dissociative reaction. It was also shown that flagella were not destroyed by the high concentration of salt. At a temperature of 55°C., which does not affect the flagella, a considerable amount of flocculating agglutinin was removed. At 65°C., which results in complete destruction of the flagella, there was no increase over the amount removed at 60°C. The latter temperature is about the critical point for the beginning destruction of flagella by heat.

The method employed was originally chosen as one favorable for the extraction of globulin. It seems probable that the liberation of flocculating agglutinin attendant upon its application is associated with that protein. Experiments bearing on the chemical nature of the extracted antibody were not attempted. While it was free in the sense that it could again unite with specific antigen it still may have retained some cellular fraction from its previous union. Locke and Hirsch (8) commenting on extraction procedures in general state that in most instances no notable dissociation of the antigen-immune substance union is produced but rather a disintegration and dispersion of the sediment, *i.e.* the sensitized antigen.

Failure to remove granulating agglutinin under the conditions of extraction might be due to differences in the chemical nature of the

two agglutinins or to differences in the manner of linkage to their respective antigens.

#### SUMMARY.

It was shown that flocculating (flagellar) agglutinin and granulating (somatic) agglutinin display certain differences with respect to their removal from sensitized bacteria (*B. paratyphi*). A 5 per cent solution of NaCl added to sedimented, sensitized bacteria followed by heating to 60°C. for 1 hour removed approximately 50 per cent of the combined agglutinin. There was little or no removal of granulating agglutinin either from the sensitized motile bacteria or from a sensitized non-motile organism (*Staphylococcus*). Evidence was presented that the agglutinin removal was not dependent solely on disintegration of flagella by the conditions of extraction with a subsequent freeing of antibody.

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## THE EFFECT OF YEAST FEEDING ON SOME BLOOD CONSTITUENTS OF HENS.

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The extensive use of yeast in various forms by mankind as a nutrient as well as a food for animals suggested the importance of studies on yeast as a constituent of the diet. As the advantages and disadvantages of a food become more evident if given in large amounts, and as the composition of the blood reflects the effect of the food on the organism, the writer undertook the following experiments of feeding hens large quantities of yeast, either alone or in addition to other ingredients.

According to Meisenheimer (23), as much as 8 per cent of the total nitrogen of yeast is in the form of purine bodies (guanine and adenine). A yeast diet may therefore cause a rise in the uric acid of the blood, as was shown experimentally by Funk, Lyle and McCaskey (7) on men.

As active yeast contains a series of powerful nucleases, it seemed of value to study the resulting differences in the blood composition due to this factor on high and low purine diets. Up to the present no attempt has been made to lower "physiologically" the per cent of purine bodies in the food by inhibiting the liberation of uric acid from nucleic bodies during the process of absorption and utilization, or by creating within the body such conditions as would stimulate the synthetic power of the corresponding nucleases to store the uric acid in the form of nucleic bodies. The purpose of the writer was to study the possibilities also in this direction.<sup>1</sup>

Hens were chosen as material for the experiments because of the ease of forced feeding and also because in the hen "the uric acid is all

<sup>1</sup> Since 1923, following the suggestion of Dr. Franklin McLean of the Peking Union Medical College, the writer has undertaken the study of the uric acid problem.

free and in the plasma" (Jones, 16). Besides, Folin and collaborators (4) said that in birds "the uric acid in the muscles bears no relation to the uric acid content of the circulating blood." Another reason was the high resistance of birds toward uric acid intoxication. In the blood of ducks with ligated ureters Folin and collaborators (4) obtained, e.g., up to 400 mgm. per cent of uric acid. Of interest is also the well-known double origin of uric acid in birds—by synthesis and by derivation from purine bodies. Kionka and Bannes (cited by Hutyra and Marek, 14) produced typical gout in birds by continuous feeding with horse flesh, which is rich in hypoxanthine. Recently Naito and Nishioka (25) found that the human organism also is capable of synthesizing uric acid from urea. Hens are known to respond to a high purine diet in the same way as mammals. It was hoped that studies on hens might help in the understanding of some obscure chapters in the uric acid problem of mammals.

**MATERIALS AND PROCEDURE.** Eighteen normal hens, Barred Plymouth Rock, weighing about 5 pounds, from 6 to 9 months old, hatched at the same time and coming from one stock, were fed the following diets:

*Two hens (nos. 6 and 7)*—semi-polished rice (a low purine diet) and water ad libitum.

*Three hens (nos. 8, 9 and 10)*—semi-polished rice and water during the first period, and semi-polished rice and diluted liquid Taka-Diastase during the second period.

*Two hens (nos. 11 and 12)*—inactive yeast (heated, Fleischmann's).

*Two hens (nos. 13 and 14)*—inactive yeast and powdered Taka-Diastase.

*Three hens (nos. 15, 16 and 17)*—active yeast (Fleischmann's).

*One hen (no. 18)*—active yeast and wheat bran.

*One hen (no. 19)*—active yeast and wheat bran the first period, and active yeast and phytin the second period.

*Two hens (nos. 20 and 21)*—active yeast the first period, and active yeast + acid sodium phosphate<sup>2</sup> the second period.

*Two hens (nos. 22 and 23)*—active yeast the first period, active yeast

<sup>2</sup> The acid sodium phosphate was given in daily doses of 0.1 gram per kilo of body weight of the hens, as such doses were found by Salvesen, Hastings and McIntosh (33) to be harmless even if given over a long period.

+ acid sodium phosphate<sup>2</sup> the second period, and active yeast + acid sodium phosphate<sup>2</sup> + cornstarch the third period.

In addition, *five hens* (nos. 1, 2, 3, 4 and 5) were fed a standard diet and served as controls.

All the hens looked healthy and strong. The droppings were mostly more or less soft.

The blood for analysis, about 40 cc, was taken at approximately the same hour after feeding. It was drawn only once from each hen from the jugular vein in oxalated tubes, centrifuged at once, and the plasma analyzed for glucose (Folin and Wu's method), chlorine

TABLE 1.

*Blood plasma of normal hens (controls) fed a standard diet\* (per 100 cc)*

Number	Sugar	N P N	Uric acid	Creatinine	Albumin	Serum globulin	Cholesterol	Chlorine	Inorganic phosphorus
	mgm	mgm	mgm	mgm	grams	grams	mgm	mgm	mgm
1	266.6	16.06	6.85	1.11	1.96	2.04	94.7	434	4.75
2	285.7	18.75	6.33	1.00	1.57	2.11	97.1	432	4.60
3	307.7	15.80	6.09	0.95	1.88	2.00	91.3	460	5.10
4	330.5	24.01	6.13	1.07	1.46	1.76	115.3	447	4.24
5	333.0	21.42	7.24	1.11	1.46	2.31	115.0	460	4.44
Average	304.7	19.21	6.53	1.05	1.67	2.04	102.7	447	4.63

\* Grain (corn, oats, wheat).

Mash (beef scrap, bran, middlings, corn meal)

Oyster shell, grit, charcoal

} ad libitum

(Whitehorn's method), non-protein nitrogen (Folin and Wu's method), uric acid (Benedict's method), creatinine (Folin and Wu's method), cholesterol (Bloor's method), inorganic phosphorus (Brigg's method), and albumin and globulin (Wu and Ling's method, 39). Details can be found in tables 1, 2 and 3.

In order to determine the presence of a nucleosidase in active yeast and Taka-Diastase, and also to determine the amount of uric acid present in inactive yeast (Fleischmann's) and Taka-Diastase, corresponding determinations were done in connection with *in vitro* experiments, as suggested by Dr. P. A. Levene of this Institute. The findings are given in table 4.

TABLE 2.  
*Blood plasma of hens fed semi-polished rice, inactive yeast and Taka-Diastase (per 100 cc.).*

Number	s.p. sex*	Inactive yeast†	Taka-diastase	Sugar mgm.	N.P.N. mgm.	Uric acid mgm.	Creatinine mgm.	Albumin gm.	Serum globulin gm.	Cholesterol mgm.	Chlorine mgm.	Inorganic phosphorus mgm.
6	27 days	—	—	235.2	16.22	6.16	1.03	2.25	1.72	150.9	411	4.54
7	Same	—	—	286.0	17.14	4.71	1.00	2.07	2.20	107.3	414	3.70
8	35 days	—	Liquid T. D.‡ for last 8 days of rice feeding	224.2	15.51	5.60	1.18	1.83	2.46	80.9	406	4.14
9	Same	—	Same	258.7	19.80	12.54	1.13	2.06	1.76	107.4	426	5.23
10	Same	—	Same	231.4	19.75	8.01	1.13	2.07	2.38	136.4	437	2.29
11	—	7 days	—	250.3	18.75	8.06	1.30	1.96	1.40	84.1	400	2.17
12	—	8 days	—	250.0	22.70	10.66	1.07	2.15	2.03	88.2	398	3.22
13	—	7 days	Powdered T. D.§ for last 6 days of yeast feeding	250.7	33.33	16.12	1.25	1.65	2.78	100.7	412	5.26
14	—	Same	Same	242.1	30.00	13.33	1.15	1.73	2.53	87.6	401	4.17

\* Ad libitum.

† 100 grams of dry yeast, moistened to form a paste, per capita per day by cramming (in two portions).

‡ Of Parke, Davis & Co. (liquid 4 per cent) diluted with 4 parts of water (0.8 per cent). Given ad libitum. Drinking water removed.

§ Of Parke, Davis & Co. (powder). Given in gelatinous capsules before yeast was fed. Average daily dose: 2.4 to 3.6 grams.

TABLE 3.  
*Blood plasma of hens fed active yeast, wheat bran, phytin, starch and NaH<sub>2</sub>PO<sub>4</sub> (per 100 cc.).*

Num- ber	Active yeast	Wheat bran	Phytin	Starch	NaH <sub>2</sub> PO <sub>4</sub>	Sugar	N P N	Uric acid	Creati- nine	Albu- min	Serum glob- ulin	Choles- terol	Chlo- rine	Inor- ganic phos- phorus
						mg. gm.	mg. gm.	mg. gm.	mg. gm.	mg. gm.	mg. gm.	mg. gm.	mg. gm.	mg. gm.
15	7 days*	—	—	—	—	250.6	23.07	13.35	1.03	1.74	3.80	72.5	404	5.17
16	14 days*	—	—	—	—	249.1	30.00	20.00	1.00	1.76	2.44	88.2	411	5.40
17	21 days*	—	—	—	—	296.3	23.24	14.02	1.07	1.91	2.50	93.8	396	5.33
18	13 days	13 days†	—	—	—	266.0	24.22	10.03	1.00	2.07	1.90	94.0	440	5.53
19	Same*	9 days†	Last 4 days of yeast feed- ing‡	—	—	400.0	35.31	17.10	1.11	1.86	1.95	75.2	397	7.90
20	18 days*	—	—	—	Last 8 days of yeast feed- ing§	250.0	35.29	15.27	0.94	2.16	2.97	187.5	336	4.84
21	Same*	—	—	—	Same	322.4	27.27	12.64	0.94	2.13	2.72	88.2	378	4.68
22	25 days*	—	—	Last 10 days of yeast feeding¶	Last 15 days of yeast feeding§	286.3	25.02	17.15	1.07	1.55	2.01	100.0	386	4.59
23	Same*	—	—	Same	Same	251.1	18.75	10.00	1.07	1.60	2.37	95.3	397	5.26

\* About 90 grams of dry yeast, moistened to form a paste, per capita per day, by cramming in two portions.

† Yeast and bran were mixed in equal parts, moistened and fed ad libitum. About 100 to 150 grams of this mixture were eaten per day.

‡ Bran was excluded. Phytin (in tablets, 3 grams daily dose) was given instead before yeast was fed (1.5 gram each time).

§ 5 cc. per capita per day of a solution (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O—4.45 grams; H<sub>2</sub>O—to 25 cc.) given in two portions mixed with the yeast (5 cc. of this solution contain 0.2 gram of P).

¶ About 20 grams of starch per capita per day, mixed with the yeast, phosphates and water to form a paste. Fed by cramming.



RESULTS. The data are given in tables 2, 3 and 4. Table 1 gives the controls. Hens 11 and 12, fed inactive yeast, show a higher albumin content than globulin. Hens 13 and 14, fed inactive yeast and Taka-Diastase, show higher globulin figures than albumin. In normal hens 1, 2, 3, 4 and 5, the per cent of globulin surpasses that of albumin. The non-protein nitrogen is high in hens 13 and 14

TABLE 4.

*The amount of uric acid\* liberated in yeast autolysate and in a lysate of inactive yeast + Taka-Diastase (calculated per 100 gram of the initial dry substance).*

Time of uric acid determination	Composition of suspensions			
	Active yeast..100 gm. Chloroform water to ..... 1 liter Toluene ..... 1 cc.†	Inactive yeast 100 gm. Taka-Diastase 10 gm. Chloroform water to ..... 1 liter Toluene .... 1 cc.†	Inactive yeast..1 gm. H <sub>2</sub> O ..... to 10 cc. Boiled at once	Taka-Diastase..1 gm H <sub>2</sub> O ..... to 10 cc. Boiled at once
	mgm.	mgm.	mgm.	mgm.
At once	—	—	26.6	40.0
24 hours	30.8	32.0	—	—
48 hours	32.0	34.8	—	—
3 days	30.8	—	—	—
4 days	—	33.3	—	—
5 days	30.8	—	—	—
8 days	—	36.4	—	—
9 days	29.6	—	—	—

\* Determined by Benedict's method in the following way: To 10 cc. of the thoroughly mixed suspension were added: 8 cc. H<sub>2</sub>O, 1 cc. Na<sub>2</sub>WO<sub>4</sub> (10 per cent) and 1 cc. H<sub>2</sub>SO<sub>4</sub> (2/3N). After mixing and keeping the suspension for 20 minutes it was filtered and 1 cc. of the filtrate taken for the determination of uric acid. Four extra cubic centimeters of H<sub>2</sub>O had to be added to it in order to avoid turbidity.

† The suspension was shaken from time to time and kept in a stoppered flask at room temperature.

fed inactive yeast and Taka-Diastase. If the corresponding figures for uric acid nitrogen  $\left( = \frac{\text{uric acid}}{3} \right)$  are subtracted from the non-protein nitrogen figures, the residual non-protein fractions remain nevertheless higher than the corresponding figures in the controls.

The inorganic phosphorus runs unusually high in hen 19, fed active

yeast and phytin, and unusually low in hens 11 and 12, fed inactive yeast. The low phosphorus of hen 10 (fed semi-polished rice and Taka-Diastase) has to be noted too. The figures for the uric acid content of plasma of our hens vary largely, depending upon the diet.

Table 4 shows that active yeast as well as Taka-Diastase is capable of liberating uric acid from nuclein bodies.

**DISCUSSION.** *Adequacy of the yeast diet.* According to Lippincott (21), the maintenance standard per day for a hen of 5 pounds weight consists of 9 grams of digestible protein, 45 grams of digestible carbohydrates and 4.5 grams of digestible fat. According to this author brewers' grains (barley refuse from the brewing of alcoholic beverages, a large portion of which is yeast) make an excellent food for hens. The analysis of Fleischmann's dry yeast, performed by Hawk, Smith and Holder (8) showed the yeast to contain 52.41 per cent of nitrogenous substance, 37.13 per cent of carbohydrates and 1.72 per cent of fat. According to Völtz (36) the protein of yeast is digested by animals to an extent of 88 per cent and the nitrogen-free extractives to 100 per cent. Osborne and Mendel (28) give for the yeast protein a digestibility (in rats) of 74 to 83 per cent.

Osborne and Mendel (28) and Meisenheimer (23) consider that the nitrogenous substances of yeast are only about 50 per cent in the form of protein, the remaining part being mostly amino acids, purine bodies (8 per cent) and ammonia. The actual protein content of yeast may therefore be taken as 25 per cent, but the amino acids may also be utilized by the organism for the synthesis of the body proteins.

According to Völtz (36), yeast contains all the nitrogenous substances which are required for all the physiological functions of the animal organism. Osborne and Mendel (28) "have kept rats successfully for more than a year, covering the period of growth, upon a diet in which yeast furnished the sole source of nitrogen as well as water-soluble vitamine." Fleischmann's inactive (heated) yeast which was used in our experiments still contains the water-soluble vitamin.

Therefore the 90 to 100 grams of dry yeast fed to the hens in the present experiments were more than adequate in protein and number of calories (although the ratio protein:carbohydrates was high). The observed changes in the blood composition of the experimental hens can therefore not be attributed to any stage of starvation.

*Phosphorus.* According to Hawk, Smith and Holder (8), Fleischmann's dry yeast contained 8.75 per cent of ash, 54.5 per cent of which was  $P_2O_5$ . It could be expected that such an enormous amount of phosphorus, mostly in the form of nucleic acid, would have greatly influenced the inorganic phosphorus content of the plasma of the experimental hens fed a yeast diet. But the data obtained on hens 15, 16 and 17, fed on active yeast, show only a slight rise in the inorganic phosphorus content of the blood. According to Levene and Medigreceanu (20), when treated with the extract of intestinal mucosa, "the yeast nucleic molecule decomposes into the following substances: phosphoric acid, purin bases, d-ribose, cytidin and uridin." It has been also recorded by many observers that the yeast nucleins suffer during the process of autolysis a complete disintegration with liberation of phosphoric acid and of free purine and pyrimidin bases. Therefore, the ingested active yeast supplies the organism with a large amount of inorganic phosphorus, the fate of which may vary.

It is of interest to note that hens 20, 21 and 22 show a much lower inorganic phosphorus content in the plasma, in spite of the same diet, consisting of active yeast, and an extra addition of phosphorus in the form of sodium diphosphate. The acid sodium phosphate might have inhibited, in our case, the action of the nucleases of the intestinal mucosa and the yeast on the nucleic acid of yeast by changing the pH (20) and as a result no phosphoric acid, or an insignificant amount, was set free. Hen 23 showed, under similar conditions, a higher inorganic phosphorus in the plasma, which is possibly due to the activity of the yeast itself in the intestinal tract resulting in the utilization of the acid sodium phosphate by the yeast cells in a medium containing starch.

At the present time very little is known about the rôle in the organism of the specific enzymes (nucleases) ingested with the food. Active or inactive (heated) yeast is fed to animals and prescribed by physicians without knowing exactly how it is going to affect the organism in toto. Our experiments with hens 11 and 12 show that when they are fed a sole diet of inactive yeast the inorganic phosphorus of the plasma drops to the abnormally low figures of 2.17 to 3.22 mgm. per cent in spite of the fact that inactive yeast still contains the same 4.4 per cent of  $P_2O_5$  as the active one. But when Taka-Dia-

stase<sup>3</sup> is added to this diet, the figures for inorganic phosphorus in the plasma of hens 13 and 14 rise to 4.17 to 5.26 mgm. per cent. Taka-Diastase contains a large number of enzymes, among which are nucleases capable of liberating the phosphoric and uric acid precursors from yeast nucleic acid, as was shown by Iwanoff (15) for the mould *Aspergillus niger* and by the writer for Taka-Diastase (see table 4). These experiments are interesting as they demonstrate the difference in the resulting effect on phosphorus metabolism between the body nucleases of the digestive tract and the foreign nucleases ingested.

*Plasma proteins.* Winterstein (38) gives for normal chicken blood serum the albumin:globulin ratio to be  $<1$  (analyzed by the  $\text{MgSO}_4$  method). By Robertson's (31) refractometric method this ratio is  $>1$ . Our experiments show that the effect of the enzymes of yeast and Taka-Diastase on the blood is not limited exclusively to the phosphate fraction. The plasma of hens 11 and 12, fed inactive yeast, shows the ratio albumin:globulin to be  $>1$ . The same ratio in hens 13 and 14, fed in addition Taka-Diastase, is  $<1$ , as is also the case in hens 15, 16 and 17, fed active yeast, and in the controls 1, 2, 3, 4 and 5. This difference can perhaps be explained by the absence of enzymes in the food of hens 11 and 12 and the presence of such in the food of the other groups. Enzymes are believed to be of protein nature, and as shown by Horvath and Chang (10) for lipase and by Pfeiffer and Standenath (30) for peptidases, they possess the capacity of penetrating through the intestinal wall into the blood stream. The writer made no direct attempt to prove the penetration of Taka-Diastase through the intestinal wall by measuring the diastatic power of the blood because Kito (17) showed that in this respect there is no difference even after the direct injection of Taka-Diastase into the blood stream. But in an indirect way the absorption of the nuclease of Taka-Diastase from the intestines was clearly demonstrated in hens 9 and 10 (see table 3) by a rise in the uric acid content of the blood on a low purine diet (of semi-polished rice) with the addition of Taka-Diastase. As the "extra" uric acid could not be derived in these hens from the food, it means that the nuclease of Taka-Diastase had penetrated through the intestinal wall and acted upon some nu-

<sup>3</sup> Taka-Diastase is the patented name of a ferment, manufactured from the *Aspergillus oryzae* mould, which is largely used in China and Japan as a yeast.

clein bodies of the organism in the tissues or the blood. The semi-polished rice contains a practically negligible amount of purine bodies, and 100 cc. of the 0.8 per cent Taka-Diastase solution contained only 3.2 mgm. of uric acid. According to Winterstein (38), the amount of blood in hens is from 3.9 to 8.7 per cent. If we take the lowest percentage (3.9) as a basis for calculation, it will show 87.7 grams of blood in a 5-pound hen. It is evident that 3.2 mgm. of uric acid are not capable of raising to a considerable extent the uric acid level in 87.7 grams of blood.

Löhr and Löhr (22) and others have found a relative increase of serum globulin after injection of protein. Foreign enzymes, as being of protein nature, may perhaps, after having penetrated into the blood from the intestines, also be capable of causing a similar rise in the globulin fraction of the blood serum, as is the case in our experiments. Immune bodies, which are believed by some authors to be of enzymatic nature, also possess the capacity of penetrating from the intestinal tract and exerting their effect on the serum globulin, as shown by T. Smith, Howe and collaborators (34), (12), (13) to be the case for colostrum. The proteins of the food (albumins or globulins) are known to have no effect on the albumin:globulin ratio of the serum (37).

*Uric acid.* According to Levene and Medigreceanu (20), the intestinal juice is capable of forming mono-nucleotides from yeast nucleins, of which the purine nucleotides are further converted into nucleosides. The extract of the intestinal mucosa is capable of setting free the purine bases. But there is no proof that they are capable of raising the uric acid figures in the blood on a low purine diet. On the other hand, foreign nucleases (yeast nucleases and Taka-Diastase) seem to possess such a capacity.

It was just mentioned that the addition of Taka-Diastase to a low purine diet resulted in hens 9 and 10 in a marked rise in the uric acid content of the blood to 12.54 and 8.01 mgm. per cent, while our figures for the normal hen on a mixed diet were 6.53 mgm. per cent. This rise cannot be attributed either to starvation (see Lennox, 18, 19) or to avitaminosis because the hens were eating rice in sufficient quantities, the rice was only semi-polished, and, besides, even complete deficiency in water-soluble vitamin does not cause any rise in the blood uric acid. Folin's (5) data for mixed normal hens' blood are 4.9

mgm. per cent (determined by the indirect method). It was shown (see table 4) that Taka-Diastase contains powerful nucleases which possess the capacity of liberating uric acid from yeast nucleic acid. The rise in the blood uric acid following the ingestion of Taka-Diastase on a low purine diet can be interpreted as a liberation of uric acid from some purine compounds of the body. This effect of Taka-Diastase may perhaps be enhanced by some amino acids present in this diastase: Lennox (18) showed that feeding asparagine (during fasting) resulted in increased elimination of uric acid without any decrease in the level of circulating uric acid. The extra uric acid came therefore from the tissues. Perhaps amino acids are capable of exerting some effect on some nuclein bodies of the tissues similar to that of an enzyme. This point of view is supported by analogous findings of Falk and Nelson (2) for lipolytic activity that amino acids are capable of hydrolyzing esters.

The observed rise in the blood uric acid of our rice-Taka-Diastase hens means that the corresponding ferment (Taka-Diastase) is capable of penetrating through the intestinal wall. This interpretation may be applied also to yeast nucleases. Hens 15, 16 and 17, of table 3, fed on active yeast, show high uric acid figures in the blood plasma (from two to three times the normal controls). The rise in the uric acid content cannot be attributed in this case to the high protein content of yeast, because, according to Folin and collaborators (4), "A high protein diet reduces the circulating level of uric acid below the levels obtainable on low protein diet."

The data for a diet of inactive (heated) yeast (hens 11 and 12, table 2) and for inactive yeast plus Taka-Diastase (hens 13 and 14) show clearly that inactive yeast causes a smaller rise in the per cent of uric acid of the blood than inactive yeast plus Taka-Diastase. It is therefore remarkable that inactive yeast, which contains the same amount of nucleins and purine bodies as active yeast, is acting physiologically as a lower purine food. Biberfeld and Schmid (1) are persuaded that free purine bases and uric acid can hardly be absorbed in large amounts from the intestinal tract. These authors quote Abderhalden and Schittenhelm, who found that the nucleic acids become in the intestines very soluble and dialysable (mono-nucleotides and nucleosides) and are evidently absorbed as such. Hence the high uric

acid content in our active yeast hens (or inactive yeast plus Taka-Diastase) might be attributed to the nucleases of yeast or Taka-Diastase absorbed from the intestines. The data for hens 15, 16 and 17 (table 3) show also that the uric acid figures are not dependent upon the length of the yeast feeding period (1, 2 or 3 weeks).

If these data, obtained on hens, would hold also for mammals, they would emphasize the enormous rôle played by "foreign" nucleases of the food, yeasts or bacteria on the plasma uric acid of other animals and man. Records of high percentages of uric acid will have to be reconsidered from this new point of view and the foods grouped not only according to their content of purine bodies (nucleins) but also in respect to the power of their nucleases. The differences in the data of various authors for uric acid of ox blood may be due partly to the enzymes of the feeds. Huttyra and Marek (14) give records of the capacity of corn smut (*Ustilago zeae*) to produce gout in hens (feeding). For cows corn smut is probably not very poisonous (Moore and T. Smith, cited by Pammel, 29). The diet of gouty individuals or of those predisposed to gout must be absolutely freed of such enzymes by cooking or other means of inactivating. The harm caused to the uric acid content by beer and wine may be due, from this point of view, chiefly to the presence of yeast nucleases. But the importance of the nucleases of the body tissues and organs must not be underestimated. Lennox (18) found that during fasting "under certain circumstances, marked changes in the concentration of uric acid in the blood were not accompanied by simultaneous and corresponding changes in uric acid output. There must be other factors (such as uricolysis or increased uric acid mobilization) to account for the facts observed." This mobilization was evidently caused by nucleases. The high blood uric acid, associated with leukemia, may be caused by the nucleases of the white blood cells. It is also known that in man, "The uric acid concentration of the blood is a delicate, if not the most delicate index of renal function at our disposal" (Myers, 24). In nephritis the elevated figures for uric acid are perhaps due not only to uric acid retention but also to some liberation of kidney nucleases into the blood stream as the result of some sort of renal obstruction. In pancreatic obstruction, for example, the lipase is absorbed into the blood stream (9). The blood uric acid figures may perhaps be lower in double nephrectomy than in animals with ligated ureters.

As the action of every enzyme can be reversed, it was hoped that perhaps favorable conditions might be created in the body to synthesize nucleic acids taking the circulating uric acid as one of its constituents. Starling (35) says in his textbook of physiology that "the nucleases . . . can certainly be synthesized by the animal." Attention must also be called to the fall in the non-protein nitrogen of the blood following the uric acid injection (Folin and collaborators, 4). It might be perhaps interpreted as a sign of some synthetic process where the uric acid and some N.P.N. constituents (amino acids?) are involved.

In order to study the possibilities of lowering the high uric acid of the blood plasma, due to active yeast feeding (continuing to feed approximately the same amount of yeast) substances rich in organic phosphorus (bran, phytin) or inorganic phosphorus (acid sodium phosphate) were given to a few hens. It was hoped that the presence of a large amount of phosphorus might inhibit the enzymatic decomposition of nucleic acid, as phosphoric acid is known to be one of its final products of cleavage. The carbohydrates of bran (starch, hexoses and pentosans), of phytin (inositol) and starch were added for the same reason. Yeast nucleic acids are known to contain a pentose (d-ribose) in their molecules, and the body nucleic acids d-ribose or a hexose.

The results were: Hen 18 (fed on active yeast and bran) showed a somewhat lower per cent of plasma uric acid, while in hen 19 (fed active yeast and phytin) the uric acid reached 17.10 mgm. per cent. Hens 20 and 21, where acid sodium phosphate was added, did not show any improvement in the uric acid content. So far the attempt has not been successful, but the work along this line will be continued.

Recently Neuberg and Kobel (27) found that amino-purines (guanine) are subject to decomposition in the presence of methylglyoxal. As the latter is a physiological product of catabolism of carbohydrates, the yeast feeding experiment with the addition of cornstarch and acid sodium phosphate was undertaken also for this reason. The results (hens 22 and 23) showed in one case a uric acid content of 17.15 mgm. per cent, and in the second 10.00 mgm. per cent. It is hard to say what caused the drop in the second case—a destruction of the purine ring in the intestines by bacteria (Rother, 32), the



decomposition in the blood by methylglyoxal or an inhibition of the action of nucleases on nucleic acid by the presence of carbohydrates and phosphates.

In general high N.P.N. figures were obtained where nucleases were present in the diet. In the case of hens 13 and 14, the 3 grams of Taka-Diastase in the food contained only 18 mgm. of N.P.N., which could not have been responsible for the rise in the blood N.P.N. The high N.P.N. fraction may be due to the albumin fraction of yeast nuclealbumins. In hens 20 and 21 the high figures for N.P.N.-uric acid are associated with very low creatinine figures. Inorganic phosphates are liberated during muscular work, and creatinine and purine bases are products of catabolism of the muscles. It is not impossible that there may exist some sparing effect of the inorganic phosphates on the destruction of more complex substances of muscular tissue. Recently Fiske and Subbarow (3) have offered proof of the presence in voluntary muscle of a compound containing one molecule each of creatine and phosphoric acid. During muscular contraction the compound evidently undergoes hydrolysis. •

#### SUMMARY AND CONCLUSIONS

1. Active yeast and Taka-Diastase possess the capacity of decomposing nucleic acid with the liberation of uric acid.
2. Feeding hens active yeast results in an increase of the plasma inorganic phosphorus and uric acid.
3. Feeding hens inactive (heated) yeast causes a drop in the plasma inorganic phosphorus and a comparatively smaller rise in uric acid.
4. The addition of Taka-Diastase to an inactive yeast ration makes it, in so far as concerns the plasma inorganic phosphorus, uric acid and protein fractions, similar to an active yeast ration.
5. The effect of a diet rich in nucleins on the inorganic phosphorus of blood plasma may depend also upon the presence of foreign nucleases in the intestines.
6. The effect on blood composition of the addition of phosphates and carbohydrates to an active yeast ration was also studied and discussed.

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## THE PREARGININE IN EDESTIN AND ITS RESISTANCE TO HYDROLYSIS.

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### I.

#### INTRODUCTION.

In a previous paper<sup>1</sup> it was shown that the amount of arginine occurring as such ( $pK' = 8.1$ ) in proteins is a small portion of the arginine found on hydrolysis. The remainder is produced by the hydrolysis of a weak basic group ( $pK' = 4.6$ ) to which the name "prearginine" is given.<sup>2</sup>

### II.

#### *Edestin.*

We now turn to edestin (a protein from hemp) which gives a high yield in arginine on hydrolysis. The available data on edestin in alkaline solution are inconsistent and we obtained new titration data in this range. Since our new data are no marked improvement over the old we will not publish them but present our analysis of the data assembled by Cohn.<sup>3</sup> Curve *A* in Fig. 1 shows the experimental values. The dotted portion of Curve *A* (in alkaline solution) indicates that the protein is modified in that range. The true curve in alkaline solution should agree with Curve *B*<sub>1</sub>.

Curve *B*<sub>1</sub> corresponds to the amino acids found on hydrolysis. In

<sup>1</sup> Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 629.

<sup>2</sup> The name "prearginine" is given to that portion of the protein molecule having a basic group ionizing about pH 4.6 and yielding arginine on hydrolysis. It may be composed of one, or of more than one, amino acid. Such a weak basic group should consist of a primary amino group attached to a conjugated unsaturated system (perhaps cyclic) as in aniline or cytosine.

<sup>3</sup> Cohn, E., *Physiol. Rev.*, 1925, v, 349.

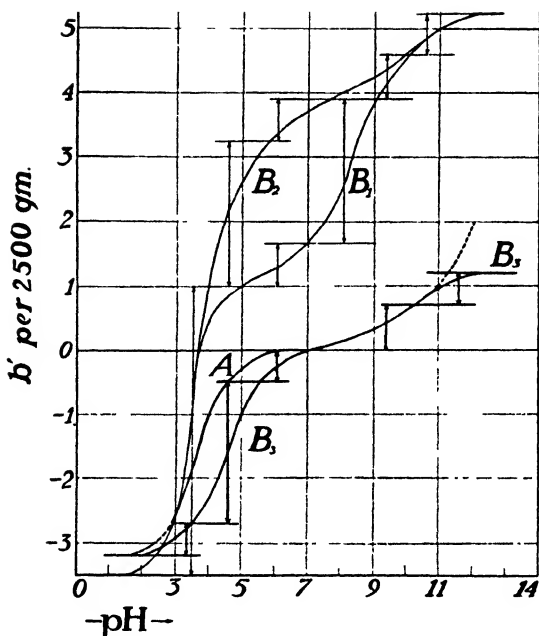


FIG. 1. A. Experimental titration curve of edestin.  $B_1$ ,  $B_2$ ,  $B_3$ , curves calculated to correspond with various amounts of the ionizable groups.

TABLE I.

*Analysis of Edestin Data Demonstrating the Relation between Arginine and Prearginine.*

Groups	Sources	Indices in edestin†	Equivalents per 2,500 gm.		
			On hydrolysis	Titration data	Difference
Acidic	Dicarboxylic acids* Tyrosine	$pG_1' = 3.0†$	4.5*	0.5	-4.0
		$pG_2' = 9.4$	0.7	0.7	0
Basic	Prearginine	$pG_3' = 3.6†$	0	2.2	+2.2
	Histidine	$pG_1' = 5.1†$	0.6	0.5	-0.5
	Arginine	$pG_4' = 8.1$	2.2	0	-2.2
	Lysine	$pG_5' = 10.6$	0.6	0.5	-0.1

\* The value for the free carboxyl groups is the total dicarboxylic acids minus the amount bound as amides ( $7.8 - 3.3 = 4.5$  equivalents).

† The indices of carboxyl groups, of prearginine and of histidine in *gelatin* are about 3.5, 4.6 and 6.1 respectively.

$B_2$ , there is no arginine but an equal amount (2.2 equivalents) of pre-arginine.  $B_3$  represents the same groups but in the amounts given in Table I (under "titration data"). This last curve ( $B_3$ ) is probably correct on the alkaline side but must be shifted to the left on the acid side in order to agree with the experimental data (Curve A). This indicates that the COOH index is a little lower than 3.5 (say 3.0) and the prearginine and histidine indices are lower than their usual values (about 3.6 and 5.1).

The results of the titration curve analysis are given in the next to the last column of Table I. The preceding column gives the equivalents of the amino acids found on hydrolysis. The values for histidine and lysine (0.6 and 0.6) are taken from Van Slyke<sup>4</sup> rather than from Vickery and Leavenworth<sup>5</sup> since the latter values (0.3 and 0.4) are lower than those we obtain from titration data (0.5 and 0.5).

It will be observed that there is *no free arginine group in edestin* and that all the 2.2 equivalents found on hydrolysis *exist in this protein as prearginine*. 4.0 equivalents of the "free" carboxyl groups do not ionize (perhaps bound as anhydride). All the other groups *i.e.*, tyrosine, histidine and lysine exist in edestin in approximately the amounts found on hydrolysis.

### III.

#### *Hydrolyzed Edestin.*

We made up four samples of edestin and hydrolyzed them with pepsin in acid solution (data given in Tables II to V). The hydrolyses were stopped at different points, neutralized with an amount of alkali exactly equal to the acid and titrated electrometrically.

Edestin contains 18.6 per cent nitrogen, or 33.3 mols per 2,500 gm. 9.0 equivalents of this is non- $\alpha$ -nitrogen in the basic groups, leaving 24.3 equivalents of  $\alpha$ -nitrogen. The initial acid-combining capacity (due to the ionizable non- $\alpha$ -nitrogen) is 3.2 equivalents. Hence the degree of hydrolysis given by the equation

$$\text{Per cent hydrolysis} = \frac{100 (A - 3.2)}{24.3}$$

<sup>4</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1911, x, 15.

<sup>5</sup> Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1928, lxxvi, 707.

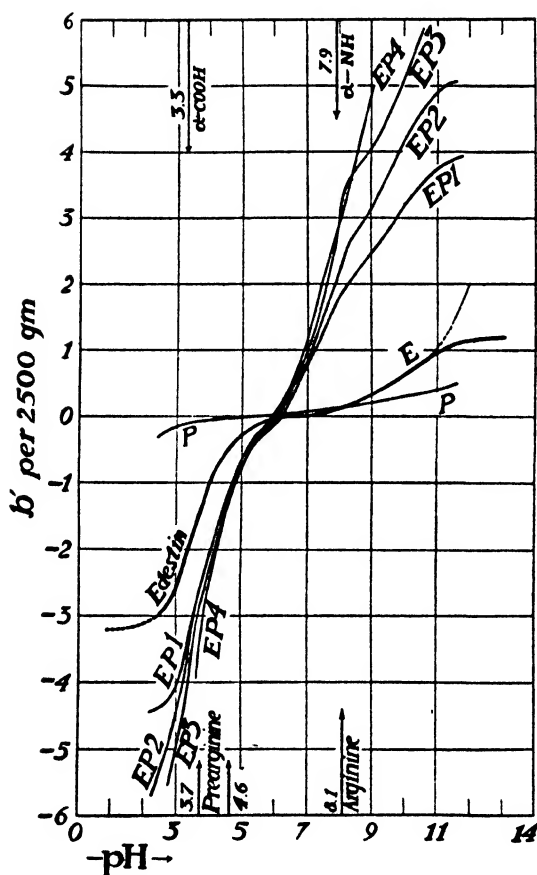


FIG. 2. Titration curves of edestin (*E*), of pepsin (*P*) and of solutions of edestin hydrolyzed to different degrees with pepsin ( $EP_1$ ,  $EP_2$ ,  $EP_3$ , and  $EP_4$ ).

where  $A$  is the acid-combining capacity (in equivalents per 2,500 gm.) of a hydrolyzed solution. On this basis the four samples of edestin were hydrolyzed 5 per cent, 14 per cent, 18 per cent and about 30 per cent respectively. These were titrated and the data are given in Tables II to V.

None of these solutions showed any change of prearginine into arginine in their titration data (given in Fig. 2). Such a change would be manifested by a drop in the  $EP$  curves at 3.7 (prearginine) and a rise at 8.1 (arginine), as compared with the Curve  $E$  of edestin. Comparison curves<sup>6</sup> were drawn and in the first three samples corroborated this conclusion (that prearginine is not converted into arginine in these solutions). However the comparison curve of the fourth sample showed that it had hydrolyzed so far that the buffer effect of the  $\alpha$  carboxyl groups (3.3) and the  $\alpha$  amino groups (7.9) would make it impossible to observe this change if it occurred.

Thus we know that prearginine is not destroyed by hydrolysis up to 18 per cent with pepsin. We also know that complete hydrolysis destroys it, since arginine can be isolated quantitatively from the resulting solution.<sup>5</sup>

Hunter<sup>7</sup> has shown that the rate of hydrolysis of edestin with trypsin indicates that arginine exists in two forms. We would suppose that the more resistant form is prearginine except that the data (Table II) indicate, in this particular protein, that there is no free arginine group (all existing as prearginine). Felix<sup>8</sup> showed that part of the "arginine" from hydrolyzed proteins was not precipitated in the usual manner. This fraction may be prearginine. Edlbacher<sup>9</sup> separated fractions high in arginine from oxidized proteins. Part of these arginine-rich fractions resisted further hydrolysis. The significance is doubtful.

<sup>6</sup> Simms, H. S., and Levene, P. A., *J. Biol. Chem.*, 1926, **lxx**, 319. The curves in Fig. 2 would be more accurate if the curve for the corresponding amount of pepsin were subtracted from the observed curve, but this would not affect the conclusions. Titration data of pepsin are given in Table VI.

<sup>7</sup> Hunter, A., *Trans. Roy. Soc. Canada*, 1925, **xix**, 1.

<sup>8</sup> Felix, K., *Z. physiol. Chem.*, 1922, **cxx**, 94.

<sup>9</sup> Edlbacher, S., *Z. physiol. Chem.*, 1924, **cxxxiv**, 129.



## IV.

*Growth-Promoting Activity.*

Dr. Lillian E. Baker kindly tested the growth-promoting activity<sup>10</sup> of the first three of the above samples of hydrolyzed edestin, on sarcomatous fibroblasts. They showed activity which was essentially the same in all three samples. The fourth sample was not tested.

## V.

## EXPERIMENTAL.

The hydrolyses of edestin were carried out as follows: 3,750 gm. edestin in about 125 cc. water, plus the designated volume of *M* HCl, plus the designated weight (0.2–0.5 gm.) of Armour's 1:10,000 pepsin was allowed to stand the designated lengths of time at 37°C.

After hydrolysis the HCl was neutralized with the same volume of *M* NaOH and the solutions were made up to 150 cc. (2.5 per cent edestin solution). The solutions were heated to 90°C. and filtered from the slight precipitate which formed on heating. These solutions were titrated by adding requisite amounts of dilute acid or base to 5 cc. samples, making up to 10 cc. and determining the pH. The final solutions were 1.25 per cent with respect to edestin, corresponding to a total volume of 300 cc.

<sup>10</sup> Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503. Baker, L. E., and Carrel, A., 1928, xlvii, 353, 371.

TABLE II.

*Titration Data of Sample EP<sub>1</sub> of Edestin Hydrolyzed with Pepsin.*

(6 cc. M HCl; 0.2 gm. pepsin; 3.5 hours at 37°C., giving 5 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	$\mu$	$b'$	pH	$\frac{b-a}{c}$	$\mu$	$b'$
2.174	-6.0	0.050	-4.448	6.3	0	0.020	0
2.580	-5.0	0.045	-4.393	6.323	0.25	0.022	0.250
3.161	-4.0	0.040	-3.842	6.663	0.50	0.023	0.500
3.294	-3.5	0.038	-3.384	6.792	0.75	0.024	0.750
3.585	-3.0	0.035	-2.941	7.316	1.0	0.025	1.000
3.819	-2.5	0.033	-2.465	7.653	1.5	0.027	1.500
4.103	-2.0	0.030	-1.982	7.936	1.75	0.029	1.750
4.381	-1.5	0.027	-1.491	8.296	2.0	0.030	2.000
4.755	-1.0	0.025	-0.996	9.061	2.5	0.033	2.497
5.007	-0.75	0.024	-0.748	9.729	3.0	0.035	2.984
5.215	-0.50	0.023	-0.500	10.941	4.0	0.040	3.739
5.472	-0.25	0.022	-0.250	11.561	5.0	0.045	3.905
6.046	-0.125	0.021	-0.125	11.827	6.0	0.050	3.965

TABLE III.

*Titration Data of Sample EP<sub>2</sub> of Edestin Hydrolyzed with Pepsin.*

(6 cc. M HCl; 0.2 gm. pepsin; 23 hours at 37°C., giving 14 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	$\mu$	$b'$	pH	$\frac{b-a}{c}$	$\mu$	$b'$
2.252	-7.0	0.055	-5.698	6.355	0.25	0.022	0.250
2.558	-6.0	0.050	-5.356	6.626	0.50	0.023	0.500
2.792	-5.0	0.045	-4.628	6.989	0.75	0.024	0.750
3.301	-4.0	0.040	-3.885	7.208	1.0	0.025	1.000
3.457	-3.0	0.035	-2.920	7.457	1.5	0.027	1.500
3.892	-2.5	0.033	-2.471	7.905	2.0	0.030	2.000
3.981	-2.0	0.030	-1.976	8.161	2.5	0.033	2.500
4.399	-1.5	0.027	-1.491	8.817	3.0	0.035	2.998
4.771	-1.0	0.025	-0.996	9.805	4.0	0.040	3.982
5.068	-0.50	0.023	-0.492	10.866	5.0	0.045	4.779
5.479	-0.25	0.022	-0.250	11.475	6.0	0.050	5.095
6.16	0	0.020	0	11.814	7.0	0.055	5.015

TABLE IV.

*Titration Data of Sample EP<sub>3</sub> of Edestin Hydrolyzed with Pepsin.*(4.5 cc. *M* HCl, 0.5 gm pepsin, 23 hours at 37°C, giving 18 per cent hydrolysis)

pH	$\frac{b-a}{c}$	$\mu$	$b$	pH	$\frac{b-a}{c}$	$\mu$	$b$
2.724	-6.0	0.052	-5.552	6.636	0.5	0.025	0.500
3.123	-5.0	0.047	-4.826	7.074	1.0	0.027	1.000
3.395	-4.0	0.042	-3.907	7.374	1.5	0.030	1.500
3.685	-3.0	0.037	-2.953	7.634	2.0	0.032	2.000
3.945	-2.5	0.035	-2.474	7.851	2.5	0.035	2.500
4.220	-2.0	0.032	-1.986	8.005	3.0	0.037	3.000
4.443	-1.5	0.030	-1.492	8.174	3.5	0.040	3.499
4.810	-1.0	0.027	-0.996	8.963	4.0	0.042	3.998
5.26	-0.5	0.025	-0.499	9.469	4.5	0.045	4.491
6.2	0	0.022	0	9.946	5.0	0.047	4.973
				10.611	6.0	0.052	5.876

TABLE V.

*Titration Data of Sample EP<sub>4</sub> of Edestin Hydrolyzed with Pepsin*(6 cc *M* HCl; 0.5 gm pepsin, 94 hours at 37°C, giving roughly 30 per cent hydrolysis)

pH	$\frac{b-a}{c}$	$\mu$	$b$	pH	$\frac{b-a}{c}$	$\mu$	$b$
3.583	-4.0	0.042	-3.948	6.575	0.50	0.025	0.500
3.869	-3.0	0.037	-2.969	6.946	1.0	0.027	1.000
4.244	-2.0	0.032	-1.987	7.480	2.0	0.032	2.000
4.846	-1.0	0.027	-0.997	8.098	3.0	0.037	3.000
5.213	-0.50	0.025	-0.499	8.544	4.0	0.042	3.999
5.614	-0.25	0.025	-0.250	9.012	5.0	0.047	4.997
5.771	0	0.022	0				

TABLE VI.

*Titration of 0.067 Per Cent Pepsin Solution*

(0.2 gm. per 150 cc mother solution or 300 cc final solution, treated like the other solutions but containing no edestin)

pH	$\frac{b-a}{c}$	$b$	pH	$\frac{b-a}{c}$	$b$
2.491	-1.0	-0.355	7.223	0.08	0.080
2.539	-0.8	-0.222	8.201	0.16	0.160
2.680	-0.6	-0.182	8.580	0.2	0.200
2.927	-0.4	-0.127	10.357	0.4	0.341
3.389	-0.2	-0.118	10.920	0.6	0.386
3.580	-0.1	-0.047	11.167	0.8	0.422
5.408	0	0	11.349	1.0	0.425
			11.631	1.6	0.500

## VI.

## SUMMARY.

The titration data of edestin show that all the arginine found on hydrolysis exists in this protein as "prearginine."<sup>1,2</sup>

The extra ionizable groups of histidine, lysine and tyrosine are free in the quantities found on hydrolysis. Part of the extra carboxyl groups of aspartic and glutamic acids are bound as amides, and 50 per cent are bound in some other manner (perhaps anhydride) leaving only about 6 per cent of these groups free to ionize in edestin.

The prearginine in edestin is not converted into arginine on hydrolysis with pepsin up to 18 per cent (of the total hydrolysis). In more highly hydrolyzed solutions it is not possible to detect such a conversion, due to high buffering. Complete hydrolysis however converts prearginine into arginine which can be isolated.

Hydrolyzed edestin promotes the growth of sarcomatous fibroblasts about equally well whether 5, 14 or 18 per cent hydrolyzed.



## CHEMICAL ANTAGONISM OF IONS.

### I. EFFECT OF Na-Mg AND K-Mg MIXTURES ON THE ACTIVITY OF OXALIC DIION.

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#### I.

#### INTRODUCTION.

It has long been known that various inorganic ions produce characteristic and specific effects on living organisms; and that in so far as the effects of two or more ions are opposite in character, they neutralize (or "antagonize") each other when present in the same solution. In this brief introductory paper it is inadvisable to review the literature on the subject, but it is sufficient to mention that interest particularly concerns four cations, namely; sodium, potassium, calcium, and magnesium.

In general the effect of sodium and potassium is to cause relaxation on one hand and greater permeability on the other. Calcium and magnesium cause contraction and impermeability. But there are also differences between the effects of sodium and potassium and between those of calcium and magnesium.

It has been found that colloids behave differently with different cations. In general calcium and magnesium precipitate sols while sodium and potassium stabilize sols. Antagonism between the effect of univalent and divalent cations on emulsions has been studied by van der Meulen and Rieman<sup>1</sup> and on arsenious sulfide sols by Weiser;<sup>2</sup>

<sup>1</sup> van der Meulen, P. A., and Rieman, W., *J. Am. Chem. Soc.*, 1924, xlii, 876.  
Rieman, W., and van der Meulen, P. A., *J. Am. Chem. Soc.*, 1925, xlvii, 2507.

<sup>2</sup> Weiser, H. B., Colloid symposium monograph, New York, 1926, iv, 354.

while Höber<sup>3</sup> and others have studied antagonism in other colloidal systems. While these are interesting, they deal with unknown factors and have failed to produce a satisfactory explanation of the biological phenomena.

The data below show that a very definite antagonism between sodium and magnesium is found in true (non-colloidal) solutions of oxalate. These agree with simple mathematical formulas. It is hoped they may throw some light on the physiological and on the colloidal antagonisms.

## II.

### Theory.

In another publication<sup>4</sup> we showed that the *monoions* of monovalent and polyvalent weak acids in the presence of  $\text{Na}^+$  or  $\text{Mg}^{++}$  ions behave normally in that they obey the limiting Debye-Hückel equation in dilute solution.

*Diions* and *triions* of weak acids in the presence of  $\text{Na}^+$  were found to obey a modified equation in which we introduced a correction for the distance between the like charges. However the presence of  $\text{Mg}^{++}$  causes marked decrease in the activity of the diion (or triion) which cannot be explained by the Debye-Hückel theory.

TABLE I.

*Inactivation of Oxalate Diion by  $\text{Mg}^{++}$ , as Shown by Conductivity Data.*

Data on 0.001 molar  $\text{MgCl}_2$  and  $\text{Na}_2\text{Ox}$  solutions and on a mixture of equal parts of these solutions. The ionic strength was equal to  $\mu = 0.003$  in all three solutions.

Solution	Conductivity $\times 10^3$	Mean
$\text{MgCl}_2$	21.1	16.5
$\text{Na}_2\text{Ox}$	11.9	
1-1 mixture	14.4	14.4
Difference.....		2.1 = 13 per cent decrease

<sup>3</sup> Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 5th edition, 1924, pp. 587-692.

<sup>4</sup> Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

The deviation of diions with  $Mg^{++}$  is small (0.8) for sebacic and azelaic acids. It is greater (1.4 and 8.3 respectively) for succinic and malonic acids, and very great (17) for oxalic acid. The effect on citric acid diion (9) and triion (29) are also large. Oxalic acid was chosen to work with owing to its high deviation and the fact that the difference between its titration indices ( $pG_2 - pG_1$ ) is large enough to greatly simplify the calculations (which are complex enough at best).

Inactivation of dianions with  $Mg^{++}$  can be shown by conductivity data, as demonstrated in Table I. The conductivity of a mixture of  $MgCl_2$  and  $NaSO_4$  solutions is 13 per cent less than the mean of the two solutions.

The antagonism experiments consisted in determining the pH of solutions of oxalic acid containing about 1.5 equivalent of NaOH to which various amounts of  $MgCl_2$  and NaCl (or KCl) were added. By calculating the  $pK'$  value and comparing it with that of a similar solution with the same ionic strength but containing NaCl alone (*i.e.*, no

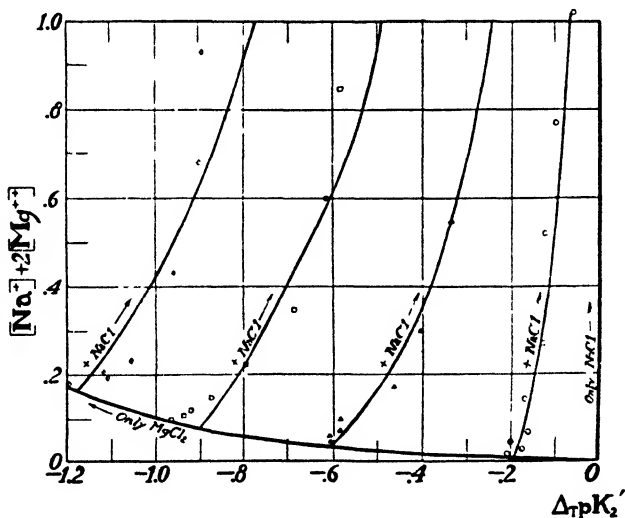


FIG. 1. Effect of  $MgCl_2$  and of  $MgCl_2$ -NaCl mixtures on  $pK_2'$  of oxalic acid (corrected for the "normal" effect produced by the same ionic strength of NaCl).  $\Delta_{Tp}K_2'$  values are equal to  $pK_2' - pK_T'$ , see Fig. 2.



$\text{MgCl}_2$ ), it was possible to determine the effect of  $\text{Mg}^{++}$  ion on the activity of the oxalate diion.

We found that with a given amount of  $\text{MgCl}_2$  the deviation was larger in the absence of  $\text{NaCl}$  or  $\text{KCl}$ . Addition of  $\text{NaCl}$  or  $\text{KCl}$  caused a decrease in this deviation, and the more  $\text{NaCl}$  or  $\text{KCl}$  added the greater the decrease until the value approached the "normal" value (with  $\text{NaCl}$  or  $\text{KCl}$  alone). Fig. 1 clearly demonstrates the effect of only  $\text{MgCl}_2$  or of  $\text{MgCl}_2 + \text{NaCl}$ .

This constitutes a definite *chemical antagonism between ions* in pure, non-colloidal solution.

### III.

#### *Formulation of the Antagonism.*

If we assume that the  $\text{Mg}^{++}$  ion combines with the oxalate diion ( $\text{Ox}^-$ ) to inactivate it and that the  $\text{Na}^+$  ion (or  $\text{K}^+$  ion) when present interferes with this combination we would expect the following equation to hold:

$$k = \frac{\text{Inactivated Ox}^-}{\text{Active Ox}^-} \times \frac{\gamma_{\text{Na}} + \gamma_{\text{K}} + 2 \gamma_{\text{Mg}}}{\gamma_{\text{Mg}}} \quad (1)$$

The experimental results show that this equation very nearly fits the facts, but that a good constant is given by the equation (where ion concentrations are substituted for activities):

$$k = \frac{\text{Inactivated Ox}^-}{\text{Active Ox}^-} \times \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}} \quad (2)$$

As will be seen in the following section the fraction of  $\text{Ox}^-$  ion inactivated by  $\text{Mg}^{++}$  is  $(1 - f')$  and that not inactivated is  $f'$ , and we may write the equation as follows:

$$k = \frac{1 - f'}{f'} \times \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}} \quad (3)$$

The introduction of the constant  $A$  (which changes with oxalate concentration) and of the concentration of oxalate diion raised to the 1.5 power, renders the equation semi-empirical rather than theoretical. The equation is nevertheless useful in proving that the observed an-

tagonism follows definite laws even down to zero concentration of  $\text{Na}^+$  and  $\text{K}^+$ .

The agreement of the data with equation (3) is summarized in Table II in which the empirical values of  $A$  are given and also the calculated values of  $k$ . The agreement of the  $k$  values may be seen in Tables III, V, VII, and VIII.

TABLE II.  
*Cation Antagonism.*

$$\text{Calculation of } k = \left( \frac{1 - f'}{f'} \right) \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox})^{1.4}}$$

Table No	Cations present	Approximate concentration of oxalate	Empirical constant $A$	$k \cdot 10^{-4}$
III	$\text{Mg}^{++}$ and $\text{Na}^+$	m/100	0.290	15
V	"	m/200	0.060	16
VII	$\text{Mg}^{++}$ and $\text{K}^+$	m/100	0.12	9
"	"	m/200	0.02	10
VIII*	$\text{Mg}^{++}$ and $\text{Na}^+$	m/100 and m/200	(0.031) and 0.023	22*
" "	$\text{Mg}^{++}$ and $\text{K}^+$	"	"	18*

\* In Tables III and V the data were obtained from solutions all containing at least 0.0075 mol of  $\text{Na}^+$  ion while the solutions in Table VII all contained  $\text{K}^+$  ions. In order to show that the equation would hold down to zero concentration of  $\text{Na}^+$  or  $\text{K}^+$  the data in Table VIII were obtained from a series including solutions free from  $\text{Na}^+$  or  $\text{K}^+$  and which were found to obey the equation. (The values of  $k$  in Table VIII are a little high, but that is unimportant.)

#### IV.

#### *Physiological Significance.*

Data in Table VIII are carried down to zero concentration of  $\text{Na}^+$  and  $\text{K}^+$ . This point is important since our experimental data are mostly in much higher concentrations of salts than the physiological concentrations. These data show that the same equations hold in the physiological range.

We have demonstrated<sup>4</sup> two types of deviations of the activity of weak electrolytes caused by the presence of  $\text{Mg}^{++}$  ions, namely the effect on weak cations and the effect on weak polyanions. We have

also shown<sup>5</sup> that  $Mg^{++}$  ions produce deviations in the activity of proteins (presumably due to both effects). Our present data show that the effect on polyanions is antagonized by  $Na^+$  and  $K^+$  ions and there should be a corresponding antagonism in protein activity.

Such an effect on protein activity may be involved in the physiological antagonisms of ions. Unfortunately we were unable to use  $Ca^{++}$  ions in our experiments due to insolubility of calcium oxalate. The effect should be similar to that with  $Mg^{++}$ . The following paper will show that a similar antagonism exists between anions ( $SO_4^-$  and  $Cl^-$ ).

We furthermore studied solutions containing  $NaCl$  and  $KCl$  (but no  $MgCl_2$ ). Although  $Na$  and  $K$  give slightly different activities, the difference is too small to demonstrate an antagonism in a mixed solution (see Table VI).

## V.

### *Experimental and Mathematical.*

A mother solution of oxalic acid was made up containing the indicated equivalents of  $NaOH$ . To 5 cc. samples of this, varying quantities of  $0.25\mu$  and  $2.5\mu$  solutions of  $NaCl$  (or  $KCl$ ) and  $MgCl_2$  were added and the solutions diluted to 10 cc. ( $0.01026$  molar oxalate). The concentrations of  $Mg^{++}$  and  $Na^+$  are given in Tables III to VIII. The concentrations of  $Na^+$  due to  $NaOH$  added are included in the indicated values for  $Na^+$ .

$b'$  was calculated from the formula:<sup>6</sup>

$$b' = \frac{b - a}{c} + \frac{h}{c} \quad (5)$$

where  $h$  is given by the concentration index (ph) which differs from the activity index (pH) by an amount varying with the ionic strength.<sup>4</sup>

$pK_s'$  is obtained by the equation:

$$pK_s' = pH - \log \frac{\alpha_2}{1 - \alpha_2} \quad (6)^{**}$$

where  $\alpha_2 = b' - 1$ .

<sup>5</sup> Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 613.

<sup>6</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

$pK_T'$  is the index of a similar solution of the same ionic strength and containing NaCl but free from  $MgCl_2$ . The  $pK_T'$  values were observed from a plot of NaCl data given in Table IV. See Fig. 2.

$\Delta_T pK_s' = pK_s' - pK_T'$  is the deviation produced by  $Mg^{++}$  ions.

The activity coefficient  $f'$  is the antilogarithm of  $\Delta_T pK_s'$ . It represents the relative amount of active diion. The actual amount

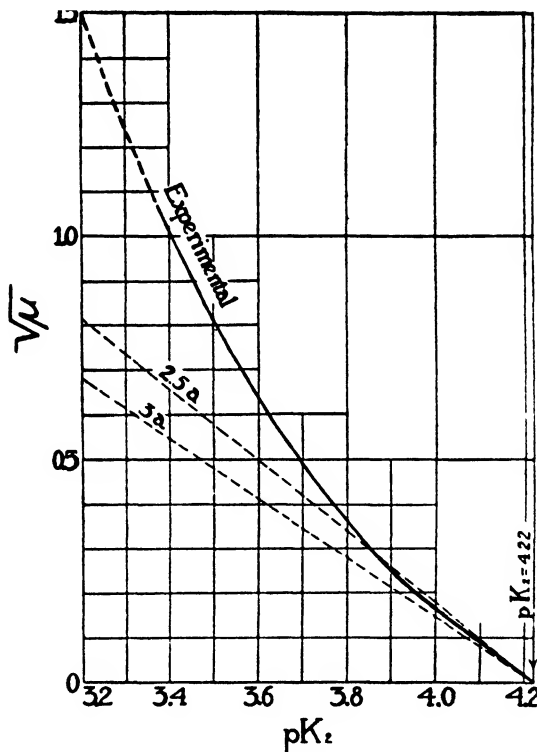


FIG. 2. Effect of NaCl on  $pK_s'$  of oxalic acid. This effect is assumed to be "normal" and the abnormalities produced by other salts are measured in terms of their deviation from this curve. Values of  $pK_s'$  read from this curve are called " $pK_T'$ ."

We have plotted the values of  $pK_s'$  against the square root of the ionic strength ( $\sqrt{\mu}$ ). Curves are also given for the limiting Debye-Hückel equation ( $pK_s - pK_s' = 3a\sqrt{\mu}$ ) and for the modified equation<sup>4</sup> ( $pK_s - pK_s' = 2.5a\sqrt{\mu}$ ).

**Average.**

[illegible]

TABLE IV.  
*Effect of NaCl Alone on  $pK_2'$  of Oxalic Acid.*

NaCl added	Na <sup>+</sup>	$\sqrt{\mu}$	pH	b'	$pK_2'$
A. (0.0126 molar oxalic acid plus 1.462 equivalents of NaOH plus varying amounts of NaCl)					
0	0.015	0.141	3.989	1.473	4.036
0.013	0.028	0.182	3.930	1.475	3.973
0.025	0.040	0.212	3.901	1.476	3.942
0.050	0.065	0.265	3.841	1.479	3.877
0.125	0.140	0.381	3.754	1.482	3.785
0.250	0.265	0.520	3.653	1.488	3.673
0.500	0.515	0.722	3.543	1.495	3.551
0.750	0.765	0.878	3.460	1.502	3.459
1.000	1.015	1.010	3.409	1.505	3.401
1.125	1.140	1.070	3.385	1.507	3.373
B. (0.00513 molar oxalic acid plus 1.462 equivalents of NaOH)					
0	0.0075	0.100	4.049	1.481	4.082
0.0025	0.0100	0.112	4.035	1.482	4.066
0.0050	0.0125	0.123	4.028	1.483	4.057
0.0075	0.0150	0.132	4.006	1.483	4.035
0.0100	0.0175	0.142	3.989	1.484	4.016
0.025	0.0325	0.187	3.945	1.487	3.967
0.050	0.057	0.245	3.883	1.492	3.896
0.075	0.082	0.292	3.861	1.494	3.870
0.100	0.107	0.332	3.832	1.496	3.835

TABLE V.

### Antagonism of $\text{Na}^+$ and $\text{Mg}^{++}$ in Dilute Solutions.

Calculation of  $k$  (where  $A = 0.060$ ) in 0.00513 molar oxalic acid plus 1.462 equivalent of NaOH with fixed amounts of  $MgCl_2$  and varying amounts of NaCl.

[illegible]



TABLE V—Continued.

[illegible]

TABLE VI.

*Effect of Mixtures of Na<sup>+</sup> and K<sup>+</sup> in Dilute Solutions.*

(0.00513 molar oxalic acid plus 1.462 equivalents of NaOH with fixed amounts of KCl and varying amounts of NaCl).

K <sup>+</sup>	Na <sup>+</sup>	$\sqrt{\mu}$	pH	b'	pK <sub>1</sub> '	pK <sub>2</sub> '	$\Delta_T pK_1'$
0.0050	0.0075	0.123	4.006	1.483	4.035	4.047	-0.012
"	0.0100	0.132	3.966	1.485	3.991	4.037	-0.046
"	0.0125	0.142	3.978	1.485	4.001	4.022	-0.021
"	0.0150	0.149	3.996	1.484	4.023	4.011	+0.022
"	0.0175	0.158	3.962	1.486	3.986	4.003	-0.017
"	0.0325	0.200	3.934	1.488	3.954	3.949	+0.005
"	0.057	0.255	3.890	1.491	3.905	3.884	+0.021
"	0.082	0.300	3.854	1.494	3.864	3.853	+0.011
"	0.107	0.339	3.817	1.497	3.822	3.819	+0.003
0.0100	0.0075	0.142	3.994	1.484	4.021	4.022	-0.001
"	0.0100	0.149	3.983	1.485	4.008	4.011	-0.003
"	0.0125	0.158	3.974	1.485	3.999	4.003	-0.004
"	0.0150	0.166	3.971	1.486	3.995	3.993	+0.002
"	0.0325	0.212	3.922	1.489	3.941	3.937	-0.004
"	0.057	0.265	3.876	1.492	3.829	3.885	-0.056
"	0.082	0.308	3.851	1.495	3.859	3.847	+0.012
0.025	0.0075	0.187	3.932	1.488	3.952	3.967	-0.015
"	0.0100	0.194	3.925	1.489	3.944	3.958	-0.014
"	0.0125	0.200	3.918	1.489	3.937	3.949	-0.012
"	0.0150	0.206	3.910	1.490	3.927	3.943	-0.016
"	0.0175	0.212	3.915	1.489	3.934	3.935	-0.001
"	0.0325	0.245	3.879	1.492	3.892	3.900	-0.008
"	0.057	0.292	3.864	1.494	3.874	3.858	+0.016
"	0.082	0.332	3.820	1.497	3.825	3.823	+0.002
"	0.107	0.367	3.803	1.499	3.804	3.794	+0.010



8 33	0 14	0 0075	0 187	3 456	1 518*	3 425	3 965	-0 540	0 288	-0 25	0 56	7 37	11
"	"	0 0100	0 194	3 470	1 516*	3 443	3 956	-0 513	0 301	-0 26	0 55	7 39	10
"	"	0 0125	0 200	3 456	1 518*	3 425	3 948	-0 532	0 294	-0 27	0 54	7 42	10
"	0 36	0 0275	0 240	3 475	1 478†	3 513	3 906	-0 393	0 404	-0 31	0 49	6 97	10
"	"	0 040	0 265	3 477	1 478†	3 515	3 885	-0 370	0 427	-0 33	0 47	7 06	9
"	"	0 065	0 308	3 494	1 478†	3 534	3 846	-0 312	0 488	-0 37	0 43	7 30	10
"	"	0 090	0 346	3 467	1 480†	3 501	3 812	-0 311	0 489	-0 41	0 39	7 39	11
"	"	0 140	0 413	3 446	1 482†	3 477	3 760	-0 283	0 521	-0 46	0 35	7 53	9
"	"	0 265	0 543	3 411	1 483†	3 437	3 654	-0 217	0 607	-0 57	0 27	7 86	9
"	"	0 515	0 738	3 357	1 491†	3 372	3 542	-0 170	0 676	-0 68	0 21	8 00	11
Average.....													10
Total average 0.00513 molar* solutions ..													10
Total average 0.01026 molar† solutions ..													9

\* 0.00513 molar oxalate,  $A = 0.020$ .† 0.01026 molar oxalate,  $A = 0.12$ .

TABLE VIII.

Antagonism of  $\text{Na}^+$  and  $\text{Mg}^{++}$  and of  $\text{K}^+$  and  $\text{Mg}^{++}$  Including Solutions with No  $\text{Na}^+$  or  $\text{K}^+$ .Calculation of  $k$  in 0.0064 molar\* solutions of oxalic acid plus 1.151 equivalents of  $\text{MgO}$  plus varying amounts of  $\text{NaCl}$  and  $\text{KCl}$ .

$\text{Mg}^{++}$	$\text{Na}^+$	$(\text{mg})^{1/2} \times 1000$	$\sqrt{\mu}$	pH	$b'$	$\text{pK}_2'$	$\text{pK}_2'$	$\Delta_{\text{pK}_2'}$	$r$	$\Delta_{\text{opK}_2}$	$f_0$	$\gamma_{\text{Mg}} \times 1000$	$k \times 10^{-4}$
0.0037	0	0.177	0.183	2.971	1.246	3.457	3.956	-0.499	0.317	-0.26	0.55	2.5	
"	0	0.070	0.129	3.191	1.263	3.638	4.042	-0.404	0.394	-0.18	0.66	3.0	22
"	0.00125	"	0.134	3.192	1.263	3.639	4.031	-0.392	0.405	-0.19	0.65	3.0	22
"	0.0025	"	0.138	3.192	1.263	3.639	4.027	-0.388	0.409	-0.19	0.64	3.1	22
"	0.0050	"	0.147	3.198	1.262	3.647	4.015	-0.368	0.429	-0.20	0.62	3.1	22
"	0.0075	"	0.156	3.206	1.260	3.659	4.008	-0.349	0.448	-0.21	0.61	3.1	22
"	0.0125	"	0.171	3.206	1.261	3.657	3.984	-0.327	0.471	-0.24	0.58	3.2	21
"	0.025	"	0.208	3.208	1.263	3.655	3.938	-0.283	0.521	-0.28	0.52	3.3	22
"	0.050	"	0.258	3.218	1.262	3.667	3.889	-0.220	0.602	-0.33	0.47	3.4	22
"	0.075	"	0.303	3.220	1.263	3.667	3.848	-0.181	0.659	-0.37	0.42	3.5	22
Average. . . . .													
22													
$\text{K}^+$													
0.0037	0	0.177	0.183	2.971	1.246	3.457	3.956	-0.499	0.317	-0.26	0.55	2.5	18
"	0	0.070	0.129	3.191	1.263	3.638	4.042	-0.404	0.394	-0.18	0.66	3.0	18
"	0.00125	"	0.134	3.191	1.263	3.638	4.031	-0.393	0.404	-0.19	0.65	3.0	18
"	0.0025	"	0.138	3.194	1.262	3.643	4.027	-0.384	0.413	-0.19	0.64	3.1	18
"	0.0050	"	0.147	3.194	1.263	3.641	4.015	-0.374	0.423	-0.20	0.62	3.1	19
"	0.0075	"	0.156	3.201	1.261	3.652	4.008	-0.346	0.451	-0.21	0.61	3.1	18
"	0.0125	"	0.171	3.211	1.260	3.665	3.984	-0.319	0.480	-0.24	0.58	3.2	18
"	0.025	"	0.208	3.218	1.260	3.672	3.938	-0.266	0.542	-0.28	0.52	3.3	18
"	0.050	"	0.258	3.233	1.258	3.692	3.887	-0.195	0.638	-0.33	0.47	3.4	18
"	0.075	"	0.303	3.243	1.257	3.704	3.848	-0.144	0.718	-0.37	0.42	3.5	16
Average. . . . .													
18													

\* The first solution of both series is 0.0128 molar ( $A = 0.031$ ) instead of 0.0064 molar ( $A = 0.023$ ).

is  $f'f \alpha_2 c$  and the actual amount of diion inactivated by  $Mg^{++}$  is  $(1 - f')f \alpha_2 c$ , hence

$$\frac{\text{Inactivated diion}}{\text{Active diion}} = \frac{(1 - f')f \alpha_2 c}{f' f \alpha_2 c} = \frac{1 - f'}{f'} \quad (7)$$

In order to correct the concentration of  $Mg^{++}$  ion for the quantity removed in inactivation we must calculate

$$\Delta_0 pK_2 = pK_T' - pK_2$$

where  $pK_2 = 4.220$  is the true index in infinite dilution.

The antilogarithm of  $\Delta_0 pK_2$  is equal to  $f$  or the activity coefficient in the presence of the same ionic strength of NaCl. We then get the activity of  $Mg^{++}$  ion from the equation:

$$\gamma_{Mg} = Mg^{++} - (1 - f')f \alpha_2 c \quad (8)$$

The value of  $(Ox^-)^{1.5}$  is equal to  $(\alpha_2 c)^{1.5}$  in the case of oxalic acid where the titration indices are isolated.

The values of  $k$  are calculated by equation (3) (see Tables II to VIII). In this equation we use concentration values of  $Na^+$ ,  $K^+$ , and  $Mg^{++}$  in the numerator rather than the activity values indicated in equation (1), the latter being unnecessary.

## VI.

### SUMMARY.

Magnesium ions decrease the activity of divalent organic anions much more than the normal decrease produced by sodium ions. The effect is very large with short chain acids, particularly oxalic acid.

The addition of sodium or potassium ions produces a marked decrease in the effect of magnesium diions on the activity of oxalate diions.

Quantitative data on 0.005 molar solutions of oxalic diion over a wide range of concentrations of  $MgCl_2$  and of NaCl (or KCl) show that the following equation is obeyed:

$$k = \frac{\text{Inactivated } Ox^-}{\text{Active } Ox^-} \times \frac{Na^+ + K^+ + 2 Mg^{++} + A}{\gamma_{Mg} (Ox^-)^{1.5}}$$

where  $A$  is an empirical value dependent on the concentration of oxalate diion ( $\text{Ox}^{2-}$ ).

This equation has been shown to hold down to zero ionic strength of  $\text{Na}^+$  and  $\text{K}^+$ , and hence to be valid in the physiological range.

These observations are of biological interest since the activity of proteins should (like oxalic acid) show a similar antagonism.

## CHEMICAL ANTAGONISM OF IONS.

### II. ANTAGONISM BETWEEN ANIONS AND ALSO BETWEEN CATIONS AND ANIONS IN THEIR EFFECT ON OXALATE ACTIVITY.

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(Accepted for publication, May 20, 1928.)

#### I.

##### INTRODUCTION.

In the previous paper<sup>1</sup> a chemical antagonism between cations was demonstrated.  $MgCl_2$  produces an anomalous depression in the activity of oxalate diions while  $NaCl$  or  $KCl$  produces a normal depression. The presence of  $NaCl$  or  $KCl$  antagonizes the anomalous effect of  $MgCl_2$  according to the semi-empirical equation:

$$k = \frac{1 - f'}{f'} \frac{2Mg^{++} + Na^+ + K^+ + A}{\gamma_{Mg} \cdot (Ox^-)^{1.5}} \quad (1)$$

where  $f'$  is the fraction of the oxalate diions ( $Ox^-$ ) inactivated by  $Mg^{++}$  and  $1-f'$  is the fraction not inactivated.

In this paper we will show that a similar behavior is displayed by  $Cl^-$  ions in antagonizing the effect of  $SO_4^{--}$  ions on oxalate ionization (an effect opposite in direction from that of  $Mg^{++}$ ). We will also show the result of having  $Mg^{++}$  and  $SO_4^{--}$  in the same solution.

#### II.

##### DISCUSSION.

Previous papers<sup>2</sup> showed that sulfates cause a rise in the values of  $pK_1'$  and  $pK_2'$  of malonic acid above the normal indices required by the limiting Debye-Hückel equation.

<sup>1</sup> Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241.

<sup>2</sup> Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.



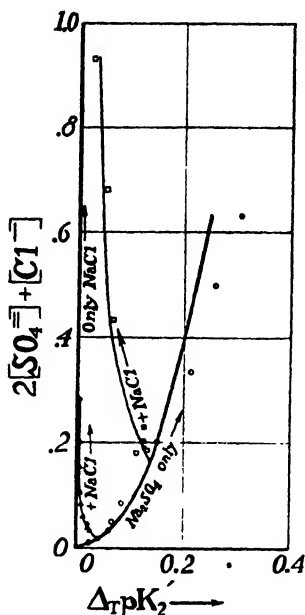


FIG. 1. Effect of  $\text{Na}_2\text{SO}_4$  and of  $\text{Na}_2\text{SO}_4$ - $\text{NaCl}$  mixtures on  $\text{pK}_2'$  of oxalic acid.

In the present study we find that the effect of  $\text{SO}_4^{2-}$  (alone) on  $\text{pK}_2'$  of oxalic acid increases with the concentration of  $\text{SO}_4^{2-}$  according to the equation:

$$k = (f' - 1) \frac{2}{\sqrt{\text{SO}_4^{2-}}} \quad (2)$$

This is shown in the right-hand curve of Fig. 1 which is drawn to fit equation (2) and agrees well with the data (circles). The value of  $k$  ( $=2.6$ ) is calculated in Table II.

The presence of  $\text{NaCl}$  antagonizes the effect of  $\text{Na}_2\text{SO}_4$ , as shown in the curves of Fig. 1 for solutions containing fixed amounts of  $\text{Na}_2\text{SO}_4$  and variable amounts of  $\text{NaCl}$ . These curves obey the equation:

$$k = (f' - 1) \frac{2\text{SO}_4^{2-} + \text{Cl}^- + A}{(\text{SO}_4^{2-})^{1.5}} \quad (3)$$

TABLE I.  
*Anion Antagonism.*

$$\text{Calculation of } k = (f' - 1) \frac{2\text{SO}_4 + \text{Cl} + A}{(\text{SO}_4)^{1.5}}$$

Table No.	Anions present*	Approximate oxalate concentration	SO <sub>4</sub> <sup>2-</sup> concentration	A	k
II	SO <sub>4</sub> <sup>2-</sup> only	m/100	0 to 0.333	0	2.6
III	SO <sub>4</sub> <sup>2-</sup> and Cl <sup>-</sup>	m/100	0.0083	0.015	2.6
			0.0833	0.015	2.6
IV	SO <sub>4</sub> <sup>2-</sup> and Cl <sup>-</sup>	m/200	0.0083	0.36	5.5
			0.0833	2.48	5.2

\* Anions present in addition to the oxalate anions.

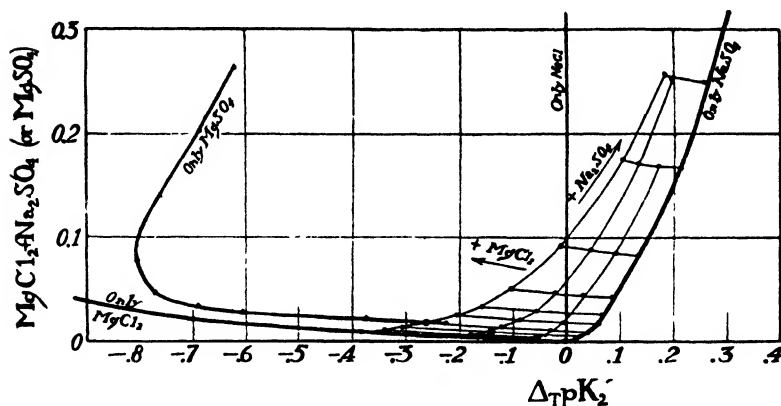


FIG. 2. Effect of Na<sub>2</sub>SO<sub>4</sub>, of MgCl<sub>2</sub>, of MgSO<sub>4</sub>, and of Na<sub>2</sub>SO<sub>4</sub> - MgCl<sub>2</sub> mixtures on pK<sub>2</sub>' of oxalic acid.

The MgSO<sub>4</sub> curve is not quantitatively comparable with the other curves due to the difference in amounts of Na<sup>+</sup> and Cl<sup>-</sup> present.

The results are summarized in Table I. It will be noted that *A* is practically zero in all m/100 solutions. However in m/200 solution of oxalate *A* varies with the sulfate concentration, and is the only case we have observed in which *A* is not a constant with a

given oxalate concentration. The experimental data are given in Tables II to IV.

The similarity between the first terms of equations (2) or (3) and that of equation (1) is shown by the relation:

$$f' - 1 = \frac{1 - 1/f'}{1/f'} \quad (4)$$

The value of  $f' = 10^{\Delta_T pK_2'}$  is less than unity in the data of equation (1), but greater than unity for the data of equations (2) and (3).

Thus we find that the effect of  $\text{SO}_4^-$  on oxalate activity is antagonized by  $\text{Cl}^-$  in a manner similar to the antagonism by  $\text{Na}^+$  or  $\text{K}^+$  of the effect of  $\text{Mg}^{++}$ .

### III.

#### *Solutions Containing Both $\text{Mg}^{++}$ and $\text{SO}_4^-$ .*

We have seen that  $\text{Mg}^{++}$  produces an effect on oxalate activity in one direction and that  $\text{SO}_4^-$  produces a similar (but smaller) effect in the opposite direction. It is interesting to study solutions containing both these anomalous ions. This is shown in Fig. 2 (data in Tables V and VI).

The heavy curves in Fig. 2 show the effect of  $\text{Na}_2\text{SO}_4$  alone, or of  $\text{MgCl}_2$  alone, or of  $\text{MgSO}_4$  alone. This last curve is seen to go sharply to the left up to 0.03 molar  $\text{MgSO}_4$ , then to turn, and above 0.1 molar point upwards to the right. At first, therefore, the effect of  $\text{Mg}^{++}$  predominates over that of  $\text{SO}_4^-$ ; but as the concentration of  $\text{MgSO}_4$  increases the effect of  $\text{SO}_4^-$  becomes relatively stronger until it predominates and tends to neutralize the initial deviation caused by  $\text{Mg}^{++}$ .

There is a close similarity between this curve for  $\text{MgSO}_4$  and the curves of LaMer<sup>3</sup> on higher valence types of other ions, an effect which he attributes to the neglect of higher terms in the Debye-Hückel equation.

We furthermore studied the effect of various proportions of  $\text{MgCl}_2$  and  $\text{Na}_2\text{SO}_4$  on oxalate activity. This effect is also shown in Fig. 2 in the lighter curves which form a network. Each curve represents

<sup>3</sup> LaMer, V. K., and Mason, C. F., *J. Am. Chem. Soc.*, 1927, xlix, 410.



#### Antagonistic Action of $\text{SO}_4^{2-}$ and $\text{Cl}^-$ on $pK_2'$ of Oxalic Acid.

Calculation of  $k$  (where  $A = 0.015$ ) in 0.01005 molar oxalic acid plus 1.520 equivalents of NaOH with fixed amounts of  $\text{Na}_2\text{SO}_4$  and varying amounts of NaCl

[illegible]



TABLE V.

*Effect of  $MgSO_4$  Alone on the Activity of Oxalic Diion.*0.01026 molar oxalic acid plus 1.462 equivalents of NaOH, plus varying amounts of  $MgSO_4$ .

$MgSO_4$	$\sqrt{\mu}$	pH	$b'$	$pK_1'$	$pK_2'$	$\Delta_2 pK_1'$
0	0.142	3.984	1.473	4.028	4.028	0
0.00313	0.180	3.722	1.483	3.751	3.976	-0.225
0.00625	0.235	3.521	1.496	3.528	3.912	-0.384
0.0125	0.265	3.311	1.518	3.280	3.883	-0.603
0.0188	0.308	3.211	1.533	3.154	3.844	-0.690
0.0313	0.381	3.106	1.554	3.014	3.784	-0.770
0.0625	0.520	3.010	1.577	2.875	3.680	-0.805
0.125	0.721	2.952	1.591	2.791	3.552	-0.761
0.188	0.878	2.939	1.592	2.777	3.464	-0.687
0.250	1.010	2.937	1.588	2.782	3.403	-0.621

TABLE VI.

*Antagonism of  $MgCl_2$  and  $Na_2SO_4$ .*(0.01005 molar oxalic acid, plus 1.520 equivalents of NaOH, with varying amounts of  $MgCl_2$  and  $Na_2SO_4$ .)

$Na_2SO_4$	0.00167 M $MgCl_2$				0.00417 M $MgCl_2$				0.00833 M $MgCl_2$			
	pH	$b'$	$pK_1'$	$\Delta_2 pK_1'$	pH	$b'$	$pK_1'$	$\Delta_2 pK_1'$	pH	$b'$	$pK_1'$	$\Delta_2 pK_1'$
0	4.003	1.531	3.950	-0.065	3.896	1.534	3.841	-0.164	3.686	1.543	3.611	-0.381
0.0017	3.994	1.531	3.941	-0.056	3.866	1.535	3.808	-0.179	3.707	1.542	3.634	-0.340
0.0042	3.983	1.532	3.928	-0.040	3.881	1.535	3.820	-0.140	3.714	1.542	3.641	-0.306
0.0083	3.957	1.533	3.900	-0.030	3.866	1.536	3.803	-0.122	3.724	1.542	3.651	-0.263
0.0167	3.937	1.534	3.878	-0.004	3.852	1.537	3.788	-0.089	3.737	1.542	3.664	-0.204
0.0250					3.844	1.537	3.780	-0.058	3.746	1.541	3.675	-0.158
0.0417	3.876	1.536	3.814	+0.030	3.805	1.539	3.757	-0.023	3.744	1.542	3.671	-0.104
0.0833	3.834	1.538	3.768	0.091	3.790	1.539	3.722	+0.046	3.734	1.542	3.661	-0.013
0.167	3.791	1.539	3.723	0.171	3.756	1.541	3.685	0.133	3.724	1.542	3.651	+0.103
0.250					3.735	1.541	3.664	0.200	3.722	1.542	3.649	0.186

## V.

## SUMMARY.

Sulfate ions ( $\text{SO}_4^-$ ) produce an anomalous effect on the ionization of oxalate diion, opposite in direction to the effect of  $\text{Mg}^{++}$  ions. This effect of sulfate is antagonized by the presence of  $\text{Cl}^-$  ions according to the equation:

$$k = (f' - 1) \frac{2\text{SO}_4^- + \text{Cl}^- + A}{(\text{SO}_4^-)^{1.5}}$$

where  $f'$  is the antilog of the increase in  $\text{pK}_2'$  due to the sulfate.

In solutions containing up to 0.03 molar  $\text{MgSO}_4$  the effect of  $\text{Mg}^{++}$  predominates over that of  $\text{SO}_4^-$ . Above 0.1 molar the effect of  $\text{SO}_4^-$  predominates and tends to neutralize the initial deviation.

In solutions containing fixed amounts of  $\text{MgCl}_2$  and varying amounts of  $\text{NaSO}_4$  (or *vice versa*) the effects of these two salts sharply antagonize each other in all proportions.





# DIFFERENCES IN SERUM AND PLASMA CONTENT OF CHOLESTEROL ESTER.

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In conducting cholesterol ester determinations in parallel on the serum and plasma of blood from the same animal, discrepancies have been observed. The cholesterol ester content of serum was invariably

TABLE I.

Animal No.	Serum		Sodium citrate plasma.		Potassium oxalate plasma.		Heparin plasma.	
	Total cholesterol	Ester cholesterol.	Total cholesterol.	Ester cholesterol.	Total cholesterol	Ester cholesterol	Total cholesterol	Ester cholesterol.
	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
1279	208	144	206	128				
1566	140	104	139	93				
5284	110	77	108	68				
13	152	87	150	55				
1414	195	119					194	119
1462	188	134					189	135
H 8	177	117			175	93	170	114
J 55	203	134			201	119	198	132
5082	197	151			185	128	185	156

greater than that of plasma while the total cholesterol values showed no differences. Since sodium citrate, 4 mg. per 1 cc. of blood, was being used as the anticoagulant in the blood from which the plasma was taken, other plasma samples were investigated in which potassium oxalate, 2 mg. per 1 cc. of blood, or heparin, 1 mg. per 5 cc. of blood, were used. The results showing the differences observed are given in Table I. All determinations were done on normal cow blood using

Bloor's (1) method for total cholesterol determinations and Bloor and Knudson's (2) for cholesterol ester determinations. All blood samples from an individual animal were drawn in succession from the jugular vein.

The data presented indicate that plasma, obtained from blood in which either sodium citrate or potassium oxalate have been used as anticoagulants, contains less cholesterol bound as ester than does serum from blood drawn from the same animal and at the same time. When heparin was used as the anticoagulant no difference in cholesterol ester content was observed between serum and heparin plasma. Although the differences found to exist between the cholesterol ester content of serum and either citrate or oxalate plasma are not very great, they are, nevertheless, of importance in that they are a source of potential error. Obviously the cholesterol ester values obtained for serum or heparin plasma more nearly represent the correct values for the non-cellular portion of the blood than do the values obtained for citrate or oxalate plasma since either sodium citrate or potassium oxalate appear to be able to cause a partial hydrolysis of that portion of the serum cholesterol generally determined as ester cholesterol.

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## CHOLESTEROL ESTERASE IN ANIMAL TISSUES.

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(Received for publication, September, 14, 1928.)

### INTRODUCTION.

From time to time evidence has been obtained in this laboratory indicating the presence in animal tissues of some very active cholesterol ester-splitting substance. The first instance of this kind was in the case of a young bull experimentally infected with *Bacillus tuberculosis*. 10 days prior to the death of this animal its total serum cholesterol was 90 mg. per cent and the serum ester cholesterol was 58 mg. per cent. As another cholesterol and cholesterol ester reading was desired, blood was taken from the heart at the time of autopsy, 2 days after the animal's death. At this time the total serum cholesterol was 69 mg. per cent while the serum ester cholesterol was 0. Other similar instances may be cited. A calf dead of an intestinal infection was autopsied 24 hours following death and blood taken from the heart at autopsy. Serum from this blood contained 144 mg. per cent total cholesterol but none was bound as ester. (Normal bovine blood serum usually contains from 50 to 70 per cent of the total cholesterol bound with fatty acid as ester.) A rabbit dying of a broken neck was bled 5 minutes post mortem. Its total serum cholesterol was 31 mg. per cent while the serum ester cholesterol was 0. 2 days earlier its total serum cholesterol had been 38 mg. per cent and the serum ester cholesterol was 23 mg. per cent. The complete disappearance of cholesterol ester from blood serum taken from an animal after death is not always observed but in the instances in which it is not observed a marked diminution is found when compared with the most recent determination made during the life of the animal.

The nature of these findings suggested two possible explanations. The first of these was that saturation of the blood with carbon dioxide

following death might cause a splitting of cholesterol ester into free cholesterol and fatty acid. The second was that the hydrolysis was due to enzyme action.

Mueller (1) in autolysis experiments with blood and mixtures of blood and liver was unable to detect any evidence of hydrolysis of cholesterol esters indicating the presence of cholesterol ester-splitting enzymes and he disputed the earlier positive results of Shultz (2) and Cytronberg (3). Porter (4) in working with esterases extracted from various animal tissues obtained evidence indicating that cholesterol esterase was present in at least a few tissues although the degree of activity was slight in most instances where it was found to be present at all. The one exception to this was in a preparation from human skin which showed quite marked cholesterol ester-splitting ability. Nomura (5) using extracts of dog, cow, horse, swine, and rabbit tissues observed an enzyme, capable of splitting synthetic cholesterol oleate, present in liver, pancreas, intestinal and gastric mucosa, spleen, kidney, and muscle but not in blood.

#### EXPERIMENTAL.

The possibility that the splitting of serum cholesterol might be due to the saturation of the blood with carbon dioxide following death was considered first.

Several samples of cow blood were drawn, in duplicate, heparin being used as the anticoagulant. Carbon dioxide was allowed to bubble slowly through one sample from each animal for half an hour. The other samples were allowed to stand at room temperature. All were then centrifuged and total and ester cholesterol determinations made on the sera. The results were entirely negative and no evidence was obtained that carbon dioxide caused any change in the serum cholesterol ester content.

The second possibility, that of enzyme action, was considered in some detail and the results obtained are given in accompanying tables.

The experimental procedure was usually as follows: Guinea pig tissues were, with one exception, used as the source of the esterase. Each tissue under test was taken from a freshly killed animal, weighed, and ground in a mortar, 5 cc. of physiological salt solution per gm. of tissue being gradually added. The mixture was then filtered through paper. The extract prepared in this way was found to contain no cholesterol. The esterase was tested against the cholesterol ester in normal cow serum. 2 cc. of salt solution tissue extract were added

to 3 cc. of cow serum in a test-tube, mixed well, and incubated at 37° for 3 hours, no preservative being used. Control tubes contained 2 cc. of physiological salt solution and 3 cc. of cow serum. One experiment was conducted in which a water suspension of cholesterol oleate was used instead of cow serum. After 3 hours incubation the total cholesterol was determined by the method of Bloor (6) and cholesterol ester by the method of Bloor and Knudson (7). The results obtained are summarized in Table I.

TABLE I.

Tissue extract.	Guinea pig tissues and cow serum.				Rabbit tissues and cow serum.		Guinea pig tissues and water suspension of cholesterol oleate.	
	Experiment 1. (Incubated 3 hrs.)		Experiment 2. (Incubated 24 hrs.)		Experiment 3. (Incubated 3 hrs.)		Experiment 4. (Incubated 3 hrs.)	
	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.	Total cholesterol.	Cholesterol ester.
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Control.....	175	127	156	79	205	170	66	61
Liver.....	174	84	159	55	198	102	72	46
Kidney.....	177	97	157	42	201	107	70	46
Muscle.....	175	98	156	54	203	117	67	52
Lung.....	177	112	153	36				
Brain.....	175	95	158	37				
Spleen.....	175	110	156	54				
Heart.....	174	114	156	54				
Testicle.....	174	108	154	72				

The exact temperature at which the cholesterol ester-splitting activity is destroyed was not determined. Boiling the salt solution tissue extract for 5 minutes completely destroyed its ability to split cholesterol ester. The temperature of inactivation lies somewhere below 67° as heating a salt solution extract of guinea pig liver at 67° for 30 minutes yielded the results given in Table II.

The degree of hydrolysis of serum cholesterol ester attained is influenced by the time that the reaction is allowed to progress and by the concentration of the salt solution tissue extract as shown by the data in Table III. In this experiment an extract of guinea pig

liver and normal cow serum were used. In one series the extract was used undiluted as in previous experiments, while in the other series it was diluted 1:4 with salt solution. Samples in each series were incubated for periods of 1, 3, 6, and 24 hours at 37°. 2 cc. of tissue extract and 3 cc. of cow serum were used as before. The control tube was incubated for 24 hours.

TABLE II.

*Guinea Pig Liver and Cow Serum. Incubated at 37° for 24 Hours.*

Tissue extract	Total cholesterol	Cholesterol ester
	mg per cent	mg per cent
Control	167	133
Liver	167	97
Liver heated to 67° for 30 min	168	132

TABLE III.

*Guinea Pig Liver and Cow Serum.*

Time of reaction	Undiluted liver extract		Liver extract diluted 1:4	
	Total cholesterol	Cholesterol ester	Total cholesterol	Cholesterol ester
hrs	mg per cent	mg per cent	mg per cent	mg per cent
1	165	116	167	119
3	168	107	167	114
6	168	104	167	105
24	167	97	168	99
Control (2 cc. salt solution and 3 cc. cow serum).	167	133		

## DISCUSSION.

The results obtained are in very close agreement with those reported previously by Nomura and are published confirmatory to those observations.

By incubating salt solution extracts of various animal tissues with blood serum or suspensions of cholesterol oleate a decrease in the cholesterol ester content of the sera or suspensions could be demon-

strated with no accompanying change in the total cholesterol. This reaction appeared to be progressive with time and dependent upon the concentration of the tissue extract. Heating the tissue extract to 67° destroyed its ability to split cholesterol ester. The ester hydrolysis is thus probably due to an enzyme that is universally present in animal tissues. All attempts to make the reaction progress to completion, as it apparently sometimes does in the animal body following death, were unsuccessful.

The fact that cholesterol esterase is present in all tissues argues that it has an important and wide-spread function. In view of the complete absence of knowledge concerning the function of cholesterol itself, surmises with regard to the nature of the rôle that cholesterol esterase plays in the animal organism would be unfruitful at this time.

Free cholesterol and cholesterol ester maintain a very constant relationship in the blood serum of a given animal over a period of time and Mueller (8) has shown that the proportion of free to combined cholesterol in the blood serum is approximately the same as that existing in the chyle during the absorption of either free cholesterol or cholesterol ester from the gastrointestinal tract. The fact that during the life of an animal a ratio between free and combined cholesterol is maintained, while soon after death the cholesterol bound as ester decreases, would indicate that the breakdown in the mechanism controlling this relationship was unequal. That is, cholesterol ester formation ceases while hydrolysis of cholesterol ester by an esterase still continues.

#### SUMMARY.

1. Blood serum obtained from an animal post mortem always contains less cholesterol ester than did serum obtained from the same animal preceding death and often contains none.

2. Passage of carbon dioxide into freshly drawn blood has no effect on the cholesterol ester content of its serum.

3. Cholesterol esterase capable of splitting either cholesterol ester in normal cow serum or cholesterol oleate in water suspension is present in many animal tissues.

4. The continued activity of cholesterol esterase post mortem with



cessation either of cholesterol ester formation or absorption may explain the complete absence or diminution of cholesterol ester in blood serum obtained from an animal after death.

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## THE HYPERCHOLESTEROLEMIA OF FASTING AS INFLUENCED BY THE SEPARATE ADMINISTRATION OF FATS, CARBOHYDRATES, AND PROTEINS.

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### INTRODUCTION.

In a previous publication (1) it was demonstrated that the high serum content of cholesterol, associated with fasting, could be rather promptly lowered by the feeding of a mixed meal to the fasting animal. At the time, the suggestion was made that the rise in serum cholesterol observed during periods of fasting might be related to the increased utilization of body fat taking place at that time and the decrease following feeding might accordingly be due to the prompt action of administered food in sparing body fat.

It seemed proper to infer that if the changes in serum cholesterol during and after fasting were truly related to some special functional connection between cholesterol and fat metabolism this would also be reflected in some difference in the influence of feeding with the separate constituents, fat, carbohydrate, or protein, to the fasting animal. This view has been subjected to the test of experiment.

A number of investigators have maintained, on the basis of indirect experimental evidence, that cholesterol has some function in fat metabolism. The data for or against this assumption may be briefly outlined.

Mayer and Schaeffer (2) found that within an animal species for any organ the proportionate content of fatty acid and cholesterol was quite constant. They termed this proportion of fatty acid to cholesterol the "lipocytic coefficient." Terroine and Weill (3) found that inanition caused wide variations in the lipid index of muscle but had little or no effect on that of the parenchymatous organs. Ingestion of food caused a transitory change in the lipid index of the liver without exerting any influence on that of other issues. The constancy of the relationship

of fats to cholesterol suggested that they were in a combination characteristic of the tissue. Terroine (4) observed that the ratio  $\frac{\text{cholesterol}}{\text{total fatty acids}}$  in dog blood was a constant for the same animal at different times and very near a constant for the species. However, he noted (5) that during fasting the constant decreased irregularly. Mayer and Schaeffer (6) found that during absolute fasting the ratio of cholesterol to fatty acids of all tissues examined, increased. Morita (7) confirmed this observation. Hueck and Wachter (8) believed that their experiments indicated that cholesterol had a prominent rôle in intermediate fat metabolism. They observed that cholesterol added to food appeared in the blood stream combined as esters with fatty acids, and also that the artificial enrichment of the diet in cholesterol not only led to a hypercholesterolemia but to an increase in the fatty acid fraction of the blood lipoids. Bloor (9) noted a relationship between fat, lecithin, and cholesterol and also between cholesterol and its esters in normal blood that was constant within narrow limits for the individual and within wider limits for the species. Anything tending to result in a disturbance of this equilibrium was followed by an attempt at restoration either by elimination or by an establishment of the relationship again on a higher or lower level. Cholesterol was found to be the most constant and least easily disturbed of the lipid constituents of normal blood. Bloor has repeatedly suggested that cholesterol has some important function in late fat metabolism (10-12). These suggestions have been based largely on the relationship found to exist between blood fat, lecithin, and cholesterol in normal and pathological human blood. Oser and Karr (13), from data obtained on normal human blood, failed to note a constant cholesterol-lecithin ratio. Terroine, Bonnet, Kopp, and Vechot (14) were unable to demonstrate any constant relationship between the aliphatic acid and sterol content of various fatty seeds or microorganisms naturally rich in fat. They did, however, obtain evidence that, in plants and microorganisms, sterols were formed at the expense of the fats. Artom (15), in a comparative study of the variation of the fatty acids and cholesterol content during artificial circulation or autolysis of the liver of a normal dog, was not able to formulate any hypothesis as to the relation between cholesterol and the fats of their cleavage products.

Experimental evidence concerning the effect of ingestion of fat upon the cholesterol content of the blood is conflicting. Beumer (16) found that during digestive lipemia an increase in serum cholesterol occurred. This increase failed to take place in a dog with a biliary fistula indicating that the increase in serum cholesterol during lipemia probably originated in the bile. Bloor (17) was unable to demonstrate any definite changes in blood cholesterol following fat ingestion. Knudson (18) fed dogs olive oil and found no constant changes in their blood cholesterol content during the resulting lipemia. He did, however, find that an increase in the ester fraction of the blood cholesterol occurred and this increase was most marked in the corpuscles, which normally are considered to contain no ester cholesterol. Chauffard, Laroche, and Grigaut (19) found that the increase in

the cholesterol content of the blood observed by them after a meal containing fat was far beyond the amount of cholesterol ingested with the food. Leites (20) fed dogs olive oil and during the resulting hyperlipemia observed an increase in the blood cholesterol, largely as free cholesterol.

## EXPERIMENTAL.

The general experimental plan was to fast animals until a definite increase in serum cholesterol content had been established. The

TABLE I.

*Effect on Fasting Hypercholesterolemia of Feeding with Pure Carbohydrate.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 13	Before fasting.	58	26	45
	After 48 hrs. without food.	105	67	64
	3 hrs. after 8 gm. glucose.	90	52	58
Rabbit 14	Before fasting.	59	24	41
	After 48 hrs. without food.	78	43	55
	3 hrs. after 8 gm. glucose.	61	26	43
Rabbit 8	Before fasting.	54		
	After 48 hrs. without food.	83		
	3 hrs. after 10 gm. cane sugar.	71		
Rabbit 17	Before fasting.	41		
	After 48 hrs. without food.	49		
	3 hrs. after 10 gm. cane sugar.	39		
Swine 390	Before fasting.	194	117	60
	After 67 hrs. without food.	240	160	67
	2 hrs. after 200 gm. glucose.	214	119	56
	6 hrs. after 200 gm. glucose.	201	117	58

fasting period was 48 hours in the case of the rabbits used and 67 hours in the case of the swine. To terminate the fast the animals were fed with either pure carbohydrates (glucose and cane sugar), proteins (gelatin and casein), or fats (olive oil and butter fat). The rabbits had to be fed by stomach tube but this was not necessary in the case of the swine. Blood samples were taken at the beginning of the period of fasting, just before feeding at the termination of the fast, and at 3 to 3½ hours following feeding in the case of the rabbits

and 2 and 6 hours in the case of the swine. Blood from the rabbits was obtained from the marginal ear vein and that from the swine was drawn from the tail. Bloor's (21) method was used for the total serum cholesterol determinations and Bloor and Knudson's (22) for the cholesterol ester determinations. The results are recorded in Tables I to III.

TABLE II.

*Effect on Fasting Hypercholesterolemia of Feeding with Pure Protein.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 12	Before fasting.	59	21	36
	After 48 hrs. without food.	86	45	52
	3 hrs. after 8 gm. gelatin.	87	45	52
Rabbit 15	Before fasting.	70	33	47
	After 48 hrs. without food.	121	63	52
	3 hrs. after 8 gm. gelatin.	124	64	52
Rabbit 66	Before fasting.	27		
	After 48 hrs. without food.	49		
	3 hrs. after 8 gm. gelatin.	54		
Rabbit 67	Before fasting.	36		
	After 48 hrs. without food.	56		
	3 hrs. after 8 gm. gelatin.	66		
Swine 392	Before fasting.	142	65	46
	After 67 hrs. without food.	164	100	61
	2 hrs. after 200 gm. casein.	165	103	62
	6 hrs. after 200 gm. casein.	145	76	52

To test further any demonstrable alteration in serum cholesterol content resulting from or accompanying an increase in fat metabolism, four rabbits were given slightly more than their caloric requirements of olive oil for a period of 2 days. They were bled at the beginning of the experiment and at the end of the 1st and 2nd days. The results obtained are given in Table IV.

#### DISCUSSION.

The separate administration of either carbohydrate, protein, or fat to an animal, deprived of food for a sufficient period of time to produce

a fasting hypercholesterolemia, resulted in all cases in a decrease in the serum cholesterol and cholesterol ester content. Carbohydrate produced perhaps the most marked and rapid decrease, due probably to the fact that it was most available to the animal in the fasting condition. There was a very definite latent period following the feeding of protein during which the serum cholesterol either remained constant or rose slightly but the observation on the swine would

TABLE III.

*Effect on Fasting Hypercholesterolemia of Feeding with Pure Fat.*

Animal No.	Time.	Total serum cholesterol.	Serum cholesterol ester.	
		mg. per cent	mg. per cent	per cent
Rabbit 18	Before fasting.	67	37	55
	After 48 hrs. without food.	86	60	70
	3½ hrs. after 5 cc. olive oil.	77	44	57
Rabbit 19	Before fasting.	50	20	40
	After 48 hrs. without food.	83	50	60
	3½ hrs. after 5 cc. olive oil.	76	46	61
Rabbit 4	Before fasting.	30		
	After 48 hrs. without food.	56		
	3½ hrs. after 5 cc. butter fat.	47		
Rabbit 62A	Before fasting.	76		
	After 48 hrs. without food.	111		
	3½ hrs. after 5 cc. butter fat.	100		
Swine 391	Before fasting.	130	51	39
	After 67 hrs. without food.	167	97	58
	2 hrs. after 100 gm. butter fat.	154	91	59
	6 hrs. after 100 gm. butter fat.	146	86	59

indicate that this was no more than transient and perhaps represented the period of time necessary for the conversion of protein into a more readily metabolizable substance. Feeding with fat produced a decrease in serum cholesterol and cholesterol ester that was only slightly less marked, as to rate and extent, than when carbohydrate had been fed. Thus these experiments, so far as they went, failed to demonstrate that the cholesterolemia of fasting could be ascribed to any disproportion in the type of food material being used by the animal

at this time as a source of energy. That is, no evidence was obtained to indicate that the fasting hypercholesterolemia was related to the increased utilization of fat taking place at this time since feeding with pure fat resulted in a lowering of the serum cholesterol level in much the same fashion as did feeding with carbohydrate, or protein, or a mixed meal. In like manner it was made quite evident that the hypercholesterolemia of fasting was related in no selective way to the metabolism of either carbohydrate or protein.

TABLE IV.

*Effect on Serum Cholesterol Level of Feeding Pure Fat to Rabbits (Olive Oil).*

Time of experiment.	Manipulation.	Rabbit 6.			Rabbit 16.			Rabbit 58.			Rabbit 62 B.		
		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.		Total serum cholesterol.	Serum cholesterol ester.	
hrs.		mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent	mg. per cent	mg. per cent	per cent
0	Bled 8 cc. All food removed from cages.	27	7	26	59	19	32	52	17	33	39	13	33
2	20 cc. olive oil.												
22	20 cc. olive oil.												
24½	Bled 8 cc.	38	10	26	58	21	36	55	18	33	46	10	22
46	20 cc. olive oil.												
48½	Bled 8 cc.	42	10	24	56	24	44	51	19	37	42	13	31

In view of the amount of indirect evidence indicating some relationship between cholesterol and fat metabolism another type of experiment was conducted, the effect of fasting upon the blood serum cholesterol content being borne in mind. Four rabbits were maintained for 48 hours on a diet of fat alone. Three of these animals failed completely to show any significant increase in serum cholesterol content while one showed a moderate rise. Observations on other rabbits, fasted for a period of 48 hours, demonstrated that, with no food at all, a very definite increase occurs. It therefore seems evident that the hypercholesterolemia of fasting is not dependent upon the

increase in fat metabolism taking place at this time and thus that cholesterol does not have a function in fat metabolism under the conditions of these experiments. If the increase serum cholesterol content of fasting could possibly be considered as bearing a relationship to the increased metabolism of fat taking place at that time then animals fasted except for their caloric requirement in fat should have been expected to show at least the degree of hypercholesterolemia that rabbits fasted for a corresponding period of time would have shown. Such was not the case for, in fact, maintenance for 48 hours on fat alone resulted in no change in serum cholesterol in any except one rabbit. This one animal showed some increase which, however, might very easily be accounted for, in the light of three negative animals, as being due to failure to utilize the fat fed with a resultant fasting hypercholesterolemia.

#### SUMMARY AND CONCLUSIONS.

1. The separate administration of either fat, carbohydrate, or protein to a fasting animal causes a decrease in the hypercholesterolemia similar to that observed following feeding with a mixed meal.

2. A diet of fat alone for 48 hours causes no significant increase in serum cholesterol.

3. The experimental evidence presented adds no weight to the assumption so often made in the past that cholesterol has a function in fat metabolism.

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## THE EFFECT OF AGE ON THE TOTAL AND COMBINED CHOLESTEROL OF THE BLOOD SERUM.

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### INTRODUCTION.

The fact that the physiological function of cholesterol and its esters has not yet been established makes it highly important to utilize every available opportunity to study its variations in the hope of obtaining evidence, which, when added to what is already known, may yield a clearer and more complete picture as to the actual rôle of cholesterol in the physiology of the animal organism. A study of the changes, in both total and combined cholesterol of the blood serum, as related to age, seemed of importance for two reasons. In the first place, it offered an opportunity to observe its variations under conditions, so far, not thoroughly studied. In the second place, in a great deal of work already in the literature, on variations in blood cholesterol as related to various disease processes or physiological phenomena, the factor of age would have vitiated the results had it been considered. It is, therefore, of importance to determine to what extent age does actually influence the cholesterol and cholesterol ester level of the blood serum.

A number of investigators have recognized that differences in blood cholesterol content occurred in organisms of differing ages or stages of development. Slemmons and Curtis (1) observed that fetal blood contained an amount of cholesterol approximately equal to the free cholesterol of the maternal blood and it was present entirely in the uncombined state. Chauffard, Laroche, and Grigaut (2) found that blood from the umbilical vein, obtained from infants immediately after birth, contained very much less cholesterol than the maternal blood. The average for four cases was found by these investigators to be 55 mg. per cent. Banu, Negresco, and Heresco (3) noted that the cholesterol content of infants' serum increased from an average of 47 mg. per cent at the age of 1 to 2 weeks to 55 mg. per cent at

the age of 8 to 10 months, both values being extremely low when compared with similar values for adult serum. György (4) observed that funicular serum contained 54 to 83 mg. per cent cholesterol whereas the maternal serum contained from 220 to 279 mg. per cent. Hellmuth (5) confirmed the observations of previous investigators concerning the low cholesterol content of new born infant blood serum but, contrary to the observation of Slemmons and Curtis, failed to find the total amount of cholesterol present in the free form. Parhon and Parhon (6) found that in extremely old people (over 70 years of age) a definite hypercholesterolemia

TABLE I.  
*Calves That Had Not Suckled Dam at Time of First Bleeding.*

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	mg. per cent	per cent
130, Holstein (female).	1 hr.	22	0	0
	48 hrs.	44	17	38
	8 days.	87	38	43
	21 "	153	69	45
	35 "	113	71	63
	57 "	112	68	61
	71 "	157	123	78
	99 "	141	116	82
	148 "	127	88	69
	199 "	93	69	74
132, Holstein (female).	1 hr.	27	0	0
	48 hrs.	48	18	38
	8 days.	102	44	43
	21 "	165	90	54
	35 "	126	76	61
	57 "	94	61	65
	71 "	122	99	82
	99 "	130	100	77
	148 "	114	84	73
	199 "	91	66	72

existed. Roffo (7) observed that in rats the blood cholesterol increased between the ages of 3 and 5 months. Baker and Carrel (8), working with chicken serum, found that the average cholesterol content of that from 3 months old chickens was 225 mg. per cent while that from 4 to 5 years old chickens was 143 mg. per cent.

#### EXPERIMENTAL.

Cattle, guinea pigs, and rabbits were the species selected for the study and the results obtained will be given separately for each species.

Bloor's method (9) was used for the total cholesterol determinations, and Bloor and Knudson's method (10) for combined cholesterol determinations. All animals used were on an adequate diet and all were bled within a period of 3 hours following feeding (new born calves and guinea pigs excepted).

### *Cattle.*

Six calves were used, blood being obtained from the external jugular vein. Two of the calves were available for only 1 day and

TABLE II.  
*Calves That Had Suckled Dam at Time of First Bleeding.*

Animal No.	Age.	Total serum cholesterol.	Combined serum cholesterol.	
		mg. per cent	mg. per cent	per cent
134. Holstein (male).	Between 5 and 6 hrs. old.	38	10	26
135. Holstein (male).	Between 5 and 6 hrs. old.	29	8	28
140, Holstein (female).	Approximately 6 hrs.	46	22	48
	48 hrs.	65	36	55
	6 days.	96	55	57
144, Jersey (female).	Approximately 6 hrs.	34	10	29
	48 hrs.	69	31	45
	6 days.	108	74	69

two others for only 6 days after birth. The remaining two calves were available for a period of approximately 7 months. The results are given in Tables I and II.

### *Guinea Pigs.*

Because of the small amount of blood that can be safely drawn from the heart of a single guinea pig, determinations were carried out on pooled samples of blood. From four to eight animals were thus used for each determination. Animals included in the groups whose ages were less than 1 month had not yet been weaned, but all other animals were on the regular diet of hay, oats, and green clover. Only male animals were used. The results obtained are given in Table III.

*Rabbits.*

Blood was obtained from the marginal ear vein in the case of the rabbits used. A small litter was chosen for the work because the animals reached a size large enough for bleeding at an earlier age. Two females and a male comprised the litter. Weaning was completed at 40 days of age. A separate litter of six animals was used in obtaining data regarding the serum cholesterol content of very young rabbits. These animals had nursed previous to the time the blood

TABLE III.  
*Guinea Pigs*

Age	Total serum cholesterol	Serum cholesterol ester		Average weight
	<i>mg per cent</i>	<i>mg per cent</i>	<i>per cent</i>	<i>gm.</i>
1 hr.	64 5	Trace.		
5 days.	231 3	124 8	53 9	
20-30 days.	63 3	28 7	45 3	136
1-2 mos.	49 6	19 3	38 9	178
2-3 "	49 8	19 9	39 9	230
3-4 "	41 3	15 0	36 3	235
4-5 "	43 2	13 7	31 7	370
5-6 "	38 1	14 6	38 3	460
6-7 "	33 8	11 9	35 2	476
7-8 "	32 8	13 3	40 5	542
8-9 "	33 3	12 0	36 0	540
9-10 "	36 0	15 5	43 0	660
11-12 "	33 3	11 5	34 5	552
16-17 "	23 8	8 7	36 5	640
18-19 "	22 5	7 6	33 7	760
24-25 "	17 0	5 8	34 1	770
29-30 "	15 0	6 3	42 0	700

sample was obtained. They were so small that it was necessary to sacrifice them in order to obtain sufficient blood. The results are given in Table IV.

## DISCUSSION.

The experimental data presented indicate that in herbivorous animals the changes in serum cholesterol as related to age are of two separate types. The first consists in a marked and rather rapid rise from birth for a relatively short period of time, varying with species,

during the early life of the animal. The second consists in a less marked and more gradual decline with advancing age. The nature of the animal material available made it impossible to observe the period of increase more than casually in any but one species of animals, cattle, while the period of decrease was observed in guinea pigs, rabbits, and cattle.

TABLE IV.

*Rabbits.*

Animal No	Age	Total serum cholesterol	Serum cholesterol ester		Weight
		mg per cent	mg per cent	per cent	gm
62 (male).	days				
	29	114 1	77 8	68 2	660
	45	96 3	53 4	55 4	1040
	65	54 0	21 3	39 4	1450
	134	42 3	5 8	13 7	2395
	162	34 0	8 3	24 4	2500
	292	20 0	9 3	46 5	2720
63 (female).	29	111 8	91 5	81 8	660
	45	104 8	63 4	60 5	1030
	65	62 6	21 7	34 6	1480
	134	76 6	38 3	50 0	3180
	162	58 6	25 6	43 7	2954
	292	52 6	28 0	53 2	3180
64 (female).	29	113 6	73 1	64 3	610
	45	95 2	41 6	43 7	1000
	65	65 0	19 0	29 2	1480
	134	75 6	19 8	26 2	3295
	162	66 0	19 1	28 9	2954
	292	55 6	26 6	47 8	3400
Pooled sample from litter of six rabbits.	3 hrs.	72 9	24 9	29 2	

The period of increasing serum cholesterol will be considered first. Blood serum obtained from calves 1 hour after birth and before any colostrum had been taken contained only free cholesterol and that in relatively small amounts. Blood serum of calves between 5 and 6 hours old already contained more cholesterol than at birth and cholesterol was present in the combined as well as the free state. All

animals bled at this time had had colostrum which in itself is quite rich in cholesterol and cholesterol ester, especially the first colostrum (11), and could very easily account for the almost immediate onset of the rise. The increase in both total and combined cholesterol was quite regularly progressive for a period of 3 weeks with the combined cholesterol increasing at a greater rate than the total cholesterol. From 3 weeks to around 10 weeks the serum cholesterol and cholesterol ester fluctuated irregularly but the ratio of combined cholesterol to total cholesterol continued to increase quite regularly. Following this and up to 200 days, the end of the experimental period, the serum cholesterol and cholesterol ester decreased quite regularly and progressively and the ratio of combined to total cholesterol decreased also. The small amount of data obtained on very young rabbits and guinea pigs indicates that these two species behave in a manner similar to cattle in that at birth the serum cholesterol content is low and rises subsequently to reach its highest level sometime under 1 month of age.

The period of declining serum cholesterol in guinea pigs and rabbits began some time under 1 month of age and was progressive and gradual. Guinea pig blood serum decreased in its cholesterol content from an average of 231 mg. per cent at 5 days of age to 15 mg. per cent in animals approximately  $2\frac{1}{2}$  years old. The cholesterol ester content of guinea pig serum decreased during the same period of time although in a less regular fashion. The per cent of total cholesterol existing in the combined form fluctuated irregularly and showed no constant changes.

The decline in serum cholesterol content in the case of rabbits was very regular for the only male animal included. Its serum cholesterol decreased from 114 mg. per cent at 29 days of age to 20 mg. per cent at 292 days of age. That the matter of sex is of some importance in determining variations in serum cholesterol with age is indicated by the results obtained with the two female rabbits. While their serum cholesterol decreased with age it did so in a rather irregular fashion and failed to reach nearly as low a level as that of their male litter mate. The combined serum cholesterol decreased with age also and the percentage in the combined form tended to diminish up until the last bleeding when it rose again in all three animals.

The observations on rabbits regarding the effect of sex in determining variations in serum cholesterol with age is supported by preliminary unpublished experiments on guinea pigs. In these it was found that female animals showed such irregular fluctuations, unrelated in any way to age, that the use of only male animals was resorted to in later experiments.

Regarding cattle, the information at hand is limited to female animals. Both Calves 130 and 132 showed the period of decreasing serum cholesterol content observed in guinea pigs and rabbits and at the age of 199 days had reached the low levels of 93 and 91 mg. per cent respectively. That irregular fluctuations in serum cholesterol content, not related to age, would have been observed in these animals had it been possible to follow them further can be stated definitely, for, in determining the cholesterol content of large numbers of samples of blood serum from lactating cows never have values below 100 mg. per cent been observed. And in the great majority of instances they were much higher. Hence, at some time after 199 days of age, factors, other than age, would undoubtedly have entered to cause marked fluctuations in the serum cholesterol content. That these factors are rather intimately associated with reproduction and lactation is indicated by the observations of Shope and Gowen (12) on variations in the serum cholesterol of cattle during the "dry" period and during early lactation. From the differences observed to exist between the sexes as regards serum cholesterol variation with age it is strongly indicated that the cholesterol in blood serum serves more functions in female than in male animals.

The significance of the variations in serum cholesterol with age is not clear because of the general haziness of our ideas concerning the possible rôles of this substance in physiology. The data presented are insufficient to indicate whether the early increase in serum cholesterol is of exogenous or endogenous origin or whether the decrease in serum cholesterol observed to take place after the preliminary rise is, conversely, a matter of decreased endogenous production or exogenous intake or both. Any discussion as to the mechanism or function of the changes observed would be little better than a surmise in the light of the deficiencies in our present knowledge concerning the physiological relationships of cholesterol.



## • SUMMARY.

1. Changes in serum cholesterol and cholesterol ester content as related to age are of two separate types. First, there is a marked and rather rapid increase from birth for a relatively short period of time during the early life of the animal. Secondly, there is a less marked and more gradual decline with advancing age.

2. At birth and before having received colostrum, sera from calves contain cholesterol only in the free form and that in very small quantities. Soon after nursing for the first time cholesterol ester appears in the blood serum and the total cholesterol content of the blood serum begins to increase.

3. Changes with age, in the serum cholesterol content, are more uniform and regular in male than in female animals.

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